Kongeriget Danmark

Patent application No.: PA 2002 01709

Date of filing: 06 November 2002

Applicants:
(Name and address)

Novo Nordisk A/S
Novo Allé
DK-2880 Bagsværd
Denmark

Title: Hidtil ukendte forbindelser.

IPC: A 61 K 38/36; C 07 K 14/745

Tis is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.

Varemærkestyrelsen
Kongeriget Danmark

Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

09 October 2003

Bo Z. Tidemann

PATENT- G VAREMÆRKESTYRELSEN

PHARMACEUTICAL COMPOSITION COMPRISING A TISSUE FACTOR ANTAGONIST AND PROTEIN C POLYPEPTIDES

FIELD OF THIS INVENTION

The present invention relates to a pharmaceutical composition comprising a TF antagonist and protein C or a protein C-related polypeptide.
The invention also relates to the use of a combination of a a TF antagonist and protein C or a protein C-related polypeptide for the manufacture of a medicament for treatment of Thrombotic or Coagulopathic related diseases, Respiratory deseases and Inflammatory diseases, or prevention hereof.

BACKGROUND OF THE INVENTION

Tissue Factor (TF) is a cellular transmembrane receptor for plasma coagulation factor VIIa and formation of TF/VIIa complexes on the cell surface triggers the coagulation cascade in vivo. The TF/VIIa complex efficiently activates coagulation factors IX and X. The resultant protease factor Xa (Xa), activates prothrombin to thrombin, which in turn converts fibrinogen into a fibrin matrix.

Normally, TF is constitutively expressed on the surface of many extravascular cell types that are not in contact with the blood, such as fibroblasts, pericytes, smooth muscle cells and epithelial cells, but not on the surface of cells that come in contact with blood, such as endothelial cells and monocytes. However, TF is also expressed in various pathophysiological conditions where it is believed to be involved in progression of disease states within cancer, inflammation, atherosclerosis and ischemia/reperfusion. Thus tissue factor is now recognised as a target for therapeutic intervention in conditions associated with increased expression.

FVIIa is a two-chain, 50 kilodalton (kDa) vitamin-K dependent, plasma serine protease which participates in the complex regulation of in vivo haemostasis. FVIIa is generated from proteolysis of a single peptide bond from its single chain zymogen, Factor VII (FVII), which is present at approximately 0.5 µg/ml in plasma. The zymogen is catalytically inactive. The conversion of zymogen FVII into the activated two-chain molecule occurs by cleavage of an internal peptide bond. In the presence of calcium ions, FVIIa binds with high affinity to exposed TF, which acts as a cofactor for FVIIa, enhancing the proteolytic activation of its substrates FVII, Factor IX and FX.

In addition to its established role as an initiator of the coagulation process, TF was recently shown to function as a mediator of intracellular activities either by interactions of the cytoplasmic domain of TF with the cytoskeleton or by supporting the VIIa-protease dependent signaling. Such activities may be responsible, at least partly, for the implicated role of TF in tumor development, metastasis and angiogenesis. Cellular exposure of TF activity is

advantageous in a crisis of vascular damage but may be fatal when exposure is sustained as it is in these various diseased states. Thus, it is critical to regulate the expression of TF activity in maintaining the health.

Inactivated FVII (FVIIai) is FVIIa modified in such a way that it is catalytically inactive. FVIIai is thus not able to catalyse the conversion of FX to FXa, but still able to bind to TF in competition with active endogenous FVIIa and thereby inhibit the TF activity.

International patent applications WO 92/15686, WO 94/27631, WO 96/12800, WO 97/47651 relates to FVIIai and the uses thereof. International patent applications WO 90/03390, WO 95/00541, WO 96/18653, and European Patent EP 500800 describe peptides derived from FVIIa having TF/FVIIa antagonist activity. International patent application WO 01/21661 relates to bivalent inhibitor of FVII and FXa.

It is often necessary to selectively block the coagulation cascade in a patient. Anticoagulants such as heparin, coumarin, derivatives of coumarin, indandione derivatives, or other agents may be used, for example, during kidney dialysis, or to treat deep vein thrombosis, disseminated intravascular coagulation (DIC), and a host of other medical disorders.

Treatment with heparin and other anticoagulants may, however, have undesirable side effects. Available anticoagulants generally act throughout the body, rather than acting specifically at a clot site. Heparin, for example, may cause heavy bleeding. Because heparin acts as a cofactor for antithrombin III (AT III), and AT III is rapidly depleted in DIC treatment, it is often difficult to maintain the proper heparin dosage, necessitating continuous monitoring of AT III and heparin levels. Heparin is also ineffective if AT III depletion is extreme. Further, prolonged use of heparin may also increase platelet aggregation and reduce platelet count, and has been implicated in the development of osteoporosis. Indandione derivatives may also have toxic side effects. In addition to the anticoagulants briefly described above, several naturally occurring proteins have been found to have anticoagulant activity, such as, for example, anticoagulant proteins isolated anticoagulant proteins from bovine aorta and human umbilical vein arteries and human placenta-derived anticoagulant proteins.

Activated Protein C is a serine protease and naturally occurring anticoagulant that plays a role in the regulation of vascular homeostasis by inactivating Factors Va and VIIIa in the coagulation cascade. Human Protein C is made in vivo primarily in the liver as a single polypeptide of 461 amino acids. In concert with other proteins, Protein C functions as an important down-regulator of blood coagulation factors that promote thrombosis. In other words, the Protein C enzyme system represents a major physiological mechanism of anticoagulation.

The critical role of protein C in controlling hemostasis is exemplified by the increased rate of thrombosis in heterozygous deficiency, protein C resistance (e.g., due to the common Fac-

tor V Leiden mutation) and the fatal outcome of untreated homozygous protein C deficiency. Human activated protein C, both plasma-derived and recombinant, have been shown to be effective and safe antithrombotic agents in a variety of animal models for both venous and arterial thrombosis. Activated protein C in recent clinical studies has been shown to be effective in human thrombotic diseases including the treatment of protein C deficiencies and microvascular thrombosis, such as disseminated intravascular coagulation associated with sepsis.

Inhibitors of tissue factor may act as antagonists for tissue factor-mediated induction of coagulation, thus blocking the production of thrombin and the subsequent deposition of fibrin. As such, TF antagonists may be useful for inhibiting tissue factor activity resulting in, for example, the inhibition of blood coagulation, thrombosis, pulmonary embolism, stroke, disseminated intravascular coagulation (DIC), platelet deposition, fibrin deposition in lungs and kidneys associated with gram-negative endotoxemia, myocardial infarction, and inflammatory responses including acute lung injury, acute respiratory distress syndrome, and systemic inflammatory response syndrome

Currently, no TF antagonists have been developed and marketed for therapeutic use in humans. Known therapeutic strategies include monoclonal antibodies, catalytically impaired FVIIa mutants and chemically inactivated FVIIa.

Therapeutic use of mouse Mabs against TF is known from U.S. patent no. 6,001,978 and 5,223,427.

International Application No. WO 99/51743 relates to human/mouse chimera monoclonal antibodies directed against human TF.

European patent application No. 833911 relates to CDR-grafted antibodies against human TF.

Presta L. et al., Thrombosis and Haemostasis, Vol. 85 (3) pp. 379-389 (2001) relates to humanized antibody against TF.

There is still a need in the art for improved compositions having anticoagulant and anti-inflammatory activity which can be administered at relatively low doses and do not produce the undesirable side effects associated with traditional anticoagulant compositions. The present invention fulfills this need by providing anticoagulants that act specifically at sites of injury or TF exposure, and further provides other related advantages. Furthermore the present invention provides compounds, which acts to inhibit the cellular functions of TF, which is implicated in conditions like sepsis, inflammation, atherosclerosis, restenosis, or cancer.

SUMMARY OF THE INVENTION

One object of the present invention is to provide compositions, which can effectively be used in the treatment or prophylaxis of Thrombotic or Coagulopathic related disease, Respiratory diseases and inflammatory diseases.

Other objects of the present invention will become apparent upon reading the present description.

In a first aspect the invention provides a pharmaceutical composition comprising a TF antagonist and protein C or a protein C-related polypeptide.

In a second aspect, the invention provides the use of a TF antagonist in combination with protein C or a protein C-related polypeptide for the manufacture of a medicament for treating Thrombotic and Coagulopathic related diseases or disorders, Respiratory diseases or disorders, and Inflammatory diseases or disorders.

In one embodiment thereof, the Thrombotic and Coagulopathic related diseases or disorders, Respiratory diseases or disorders, and Inflammatory diseases or disorders include deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, tumour metastasis, inflammation, septic chock, hypotension, acute lung injury (ALI), Acute Respiratory Distress Syndromr (ARDS), pulmonary embolism, disseminated intravascular coagulation (DIC), sepsis, systemic inflammatory response syndrome (SIRS), vascular restenosis, platelet deposition, myocardial infarction, angiogenesis, or the prophylactic treatment of mammals with atherosclerotic vessels at risk for thrombosis; asthma, bronchitis, idiopathic pulmonary fibrosis, pneumonia, pulmonary edema, pulmonary obstructive disease, endotoxin induced lung damage, non cell lung cancer; inflammatory bowel disease, pancreatitis, trauma-induced shock, bronchial asthma, allergic rhinitis, rheumatoid arthritis, cystic fibrosis, stroke, acute bronchitis, chronic bronchitis, acute bronchiolitis, chronic bronchiolitis, osteoarthritis, gout, spondylarthropathris, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, enterapathric spondylitis, juvenile arthropathy or juvenile ankylosing spondylitis, reactive arthropathy, infectious or post-infectious arthritis, gonoccocal arthritis, tuberculous arthritis, viral arthritis, fungal arthritis, syphilitic arthritis, Lyme disease, arthritis associated with "vasculitic syndromes," polyarteritis nodosa, hypersensitivity vasculitis, Luegenec's granulomatosis, polymyalgin rheumatica, joint cell arteritis, calcium crystal deposition arthropathris, pseudo gout, non-articular rheumatism, bursitis, tenosynomitis, epicondylitis (tennis elbow), carpal tunnel syndrome, repetitive use injury (typing), miscellaneous forms of arthritis, neuropathic joint disease (charco and joint), hemarthrosis (hemarthrosic), Henoch-Schonlein Purpura, hypertrophic osteoarthropathy, multicentric reticulohistiocytosis, arthritis associated with certain diseases, surcoilosis, hemochromatosis, sickle cell disease and other hemoglobinopathries, hyperlipoproteineimia, hypogammaglobulinemia, hyperparathyroidism, ac-

romegaly, familial Mediterranean fever, Behat's Disease, systemic lupus erythrematosis, relapsing, and multiple organ failure resulting from any of the preceding pathologic processes.

In one embodiment, the diseases or disorders are Respiratory disease and Inflammatory disease. In one embodiment, Respiratory disease and Inflammatory disease include lower respiratory diseases such as systemic inflammatory response syndrome, asthma, bronchitis, acute lung injury, acute resporatory distress syndrome, idiopathic pulmonary fibrosis, pneumonia, pulmonary edema, pulmonary obstructive disease, endotoxin induced lung damage, non cell lung cancer; inflammatory bowel disease, sepsis, septic shock, acute respiratory distress syndrome, pancreatitis, trauma-induced shock, bronchial asthma, allergic rhinitis, rheumatoid arthritis, cystic fibrosis, stroke, acute bronchitis, chronic bronchitis, acute bronchiolitis, chronic bronchiolitis, osteoarthritis, gout, spondylarthropathris, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, enterapathric spondylitis, juvenile arthropathy or juvenile ankylosing spondylitis, reactive arthropathy, infectious or post-infectious arthritis, gonoccocal arthritis, tuberculous arthritis, viral arthritis, fungal arthritis, syphilitic arthritis, Lyme disease, arthritis associated with "vasculitic syndromes," polyarteritis nodosa, hypersensitivity vasculitis, Luegenec's granulomatosis, polymyalgin rheumatica, joint cell arteritis, calcium crystal deposition arthropathris, pseudo gout, non-articular rheumatism, bursitis, tenosynomitis, epicondylitis (tennis elbow), carpal tunnel syndrome, repetitive use injury (typing), miscellaneous forms of arthritis, neuropathic joint disease (charco and joint), hemarthrosis (hemarthrosic), Henoch-Schonlein Purpura, hypertrophic osteoarthropathy, multicentric reticulohistiocytosis, arthritis associated with certain diseases, surcoilosis, hemochromatosis, sickle cell disease and other hemoglobinopathries, hyperlipoproteineimia, hypogammaglobulinemia, hyperparathyroidism, acromegaly, familial Mediterranean fever, Behat's Disease, systemic lupus erythrematosis, relapsing, and multiple organ failure resulting from any of the preceding pathologic processes.

In another embodiment, the diseases or disorders are Thrombotic or Coagulopatic related diseases or disorders. In one embodiment, Thrombotic or Coagulopatic related disease include vascular diseases and inflammatory responses such as deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, tumour metastasis, inflammation, septic chock, hypotension, acute lung injury (ALI), Acute Respiratory Distress Syndromr (ARDS), pulmonary embolism, disseminated intravascular coagulation (DIC), sepsis, systemic inflammatory response syndrome (SIRS), vascular restenosis, platelet deposition, myocardial infarction, angiogenesis, or the treatment of mammals with atherosclerotic vessels at risk for thrombosis, and multiple organ failure resulting from any of the preceding pathologic processes.

In a preferred embodiment, the disease or disorder is one or more of systemic inflammatory response syndrome, acute lung injury, acute respiratory distress syndrome, disseminated

intravascular coagulation, sepsis, or multiple organ failure in association with any of the preceeding syndromes.

In another embodiment, the medicament is formulated for intravenous administration, preferably injection or infusion, in particular injection.

In one embodiment, the medicament is formulated in single-unit dosage form; in another it is formulated in the form of a first unit dosage form comprising a preparation of a TF antagonist and a second unit dosage form comprising a preparation of protein C or a protein C-related polypeptide.

In a further aspect, the invention provides a method for treating Thrombotic or Coagulopathic related disease, Repiratory disease and Inflammatory disease in a subject, the method comprising administering to a subject in need thereof a first amount of a preparation of a TF antagonist and a second amount of a preparation of protein C or a protein C-related polypeptide, wherein the first and second amount together are effective to treat Thrombotic or Coagulopathic related disease, Repiratory disease and Inflammatory disease.

In one embodiment the TF antagonist and the protein C or protein C-related polypeptide are present in a ratio by mass of between about 100:1 and about 1:100 (w/w factor VII:protein C).

In another embodiment, the pharmaceutical composition is formulated for intravenous administration, preferably injection or infusion, in particular injection. In one embodiment, the composition contains at least one pharmaceutical acceptable excipients or carrier.

In one embodiment of the invention, the TF antagonist and the protein C or protein C-related polypeptide are administered simultaneously. In another embodiment, the TF antagonist and the protein C or protein C-related polypeptide are administered sequentially.

In one embodiment of the present invention, the pharmaceutical composition is in single-dosage form and consists essentially of a preparation of a TF antagonist and a preparation of protein C or a protein C-related polypeptide, and one or more of the components selected from the list of pharmaceutical acceptable excipients or carriers, stabilizers, detergents, neutral salts, antioxidants, preservatives, and protease inhibitors. In another embodiment, the pharmaceutical composition is in the form of a first-unit dosage form and a second-unit dosage form, where the first-unit dosage form consists essentially of a preparation of a TF antagonist and one or more of the components selected from the list of pharmaceutical acceptable excipients or carriers, stabilizers, detergents, neutral salts, antioxidants, preservatives, and protease inhibitors; and the second-unit dosage form consists essentially of a preparation of protein C or a protein C-related polypeptide and one or more of the components selected from the list of pharmaceutical acceptable excipients or carriers, stabilizers, detergents, neutral salts, antioxidants, preservatives, and protease inhibitors.

In one embodiment of the present invention the TF antagonist is factor VII polypeptides chemically inactivated in the active site. In one embodiment of the present invention the TF antagonist is an antibody against TF. In one embodiment the antibody is a monoclonal antibody. In one embodiment the antibody is a human monoclonal antibody. In one embodiment the antibody is an antibody against human TF. In one embodiment, the TF antagonist is selected from a list of: a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH I domains; a F(ab)2 or F(ab')2 fragment; a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the VH and CH1 domains; a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, a dAb fragment; an isolated complementarity determining region (CDR); and single chain Fv (scFv).

In one embodiment, the TF antagonist is selected from a list consisting of human and bovine Factor VII, wherein the active site residue Ser344 is modified, replaced with Gly, Met, Thr, or more preferably, Ala. Such substitution can be made separately or in combination with substitution(s) at other sites in the catalytic triad, which includes His193 and Asp242.

In one embodiment of the invention, the TF antagonist is human factor VII, which has an amino acid substitution of the lysine corresponding to position 341 of SEQ ID NO: 1.

In one embodiment of the invention, he TF antagonist is human factor VII, which has an amino acid substitution of the serine corresponding to position 344 of SEQ ID NO: 1.

In one embodiment of the invention, the TF antagonist is human factor VII, which has an amino acid substitution of the aspartic acid corresponding to position 242 of SEQ ID NO: 1.

In one embodiment of the invention, the TF antagonist is human factor VII, which has an amino acid substitution of the histidine corresponding to position 193 of SEQ ID NO: 1.

In one embodiment, the TF antagonist is a factor VII polypeptide selected from the list consisting of: FVII-(K341A), FVII-(S344A), FVII-(D242A) and FVII-(H193A).

In one embodiment, the TF antagonist is a factor VII polypeptide inactivated in the active site by reaction with a reagent selected from the list of: peptide chloromethylketones or peptidyl cloromethanes; azapeptides; acylating agents such as various guanidinobenzoate derivatives and 3-alkoxy-4-chloroisocoumarins; sulphonyl fluorides such as phenylmethylsulphonylfluoride (PMSF); diisopropylfluorophosphate (DFP); tosylpropylchloromethyl ketone (TPCK); tosylysylchloromethyl ketone (TLCK); nitrophenylsulphonates; heterocyclic protease inhibitors such as isocoumarines, and coumarins.

In one embodiment, the TF antagonist is a factor VII polypeptide inactivated in the active site by reaction with a reagent selected from the list of: Phe-Phe-Arg chloromethyl ketone, Phe-Phe-Arg chloromethylketone, D-Phe-Phe-Arg chloromethyl ketone, D-Phe-Phe-Arg chloromethylketone Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, L-Glu-Gly-Arg chloromethylketone and D-Glu-Gly-Arg chloromethylketone, Dansyl-Phe-Phe-Arg chloromethyl

ketone, Dansyl-Phe-Phe-Arg chloromethylketone, Dansyl-D-Phe-Phe-Arg chloromethyl ketone, Dansyl-D-Phe-Phe-Arg chloromethylketone, Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-D-Phe-Pro-Arg chloromethylketone, Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-D-Phe-Pro-Arg chloromethylketone, Dansyl-L-Glu-Gly-Arg chloromethylketone and Dansyl-D-Glu-Gly-Arg chloromethylketone.

In one embodiment, the protein C polyeptide is protein C; in another embodiment, the protein C polypeptide is human protein C; in another embodiment, the protein C polypeptide is a protein C-related polypeptide; in one embodiment, the protein C polypeptide is a protein C sequence variant; in one embodiment, the protein C polypeptide is in its zymogen form, in another embodiment, the protein C polypeptide is in its activated form. In one embodiment, the protein C polypeptide is recombinantly made. In preferred embodiments, the protein C polypeptide is recombinant human protein C or recombinant activated human protein C.

DETAILED DESCRIPTION OF THIS INVENTION

Tissue factor antagonists

The terms "TF antagonist" or "TF antagonists", as used herein is intended to mean any compound binding directly to TF and inhibiting the activation, or conversion, of factor X to factor Xa. In practising the present invention, any such compound binding directly to tissue factor and inhibiting conversion of factor X to factor Xa may be used. This includes, without limitation, factor VII polypeptides having substantially reduced catalytic activity, inhibitory antibodies against TF, as well as fragments thereof. The TF antagonists bind to tissue factor with high affinity and specificity but do not initiate blood coagulation.

In one embodiment of the invention, TF antagonists encompass those that exhibit at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 100%, at least about 110%, at least about 120%, or at least about 130%, of the specific TF-binding affinity of wild-type factor VIIa, when tested in one or more of the TF binding assays as described in the present specification. In a preferred embodiment, the TF antagonists exhibit at least about 75% of the binding affinity of wild-type factor VIIa. The term "TF binding activity" as used herein means the ability of a FVIIa polypeptide or TF antagonist to inhibit the binding of recombinant human 125I-FVIIa to cell surface human TF. The TF binding activity may be measured as described in Assay 3 (of the present specification).

In another embodiment, TF antagonists encompass those that exhibit less than about 50%, preferably less than about 25%, more preferably less than about 10%, or 5%, or 3%, or 2%, and most preferably less than about 1% of the specific activity of wild-type factor VIIa,

when tested in one or more of a clotting assay, or proteolysis assay as described in the present specification.

The TF antagonists for use in the present invention include, without limitation, immunoglobulin molecules and fragments thereof that have the ability to specifically bind to an antigen (i.e., TF) such as (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH I domains; (ii) F(ab)2 and F(ab')2 fragments; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment; (vi) an isolated complementarity determining region (CDR); (vii) single chain Fv (scFv); and (viii) diabodies. Included are also antibodies having variable and constant regions derived from human germline immunoglobulin sequences; human antibodies including amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), for example in the CDRs and in particular CDR3; antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences, e.g. the so-called humanized antibodies or human/mouse chimera antibodies.

The TF antagonists for use in the present invention also encompass, without limitation, factor VII polypeptides that has substantially reduced ability to catalyse the conversion of factor X to factor Xa ("inactivated" factor VII polypeptides).

The terms "FVII polypeptide" or "FVII polypeptides" as used herein include, without limitation, native Factor VII, as well as factor VII-related polypeptides that have either been chemically modified relative to human factor VII and/or contain one or more amino acid sequence alterations relative to native Factor VII (i.e., Factor VII variants), and/or contain truncated amino acid sequences relative to native Factor VII (i.e., Factor VII fragments).

It further encompasses polypeptides with a slightly modified amino acid sequence, for instance, polypeptides having a modified N-terminal end including N-terminal amino acid deletions or additions, and/or polypeptides that have been chemically modified relative to human factor VIIa. Such factor VII-related polypeptides may exhibit different properties relative to native Factor VII, including stability, phospholipid binding, altered specific proteolytic activity, and the like. Factor VII-related polypeptides also include proteolytically inactive variants of Factor VII.

The terms "variant" or "variants", as used herein, is intended to designate human Factor VII having the sequence of SEQ ID NO: 1, wherein one or more amino acids of the parent protein have been substituted by another amino acid and/or wherein one or more amino acids of the parent protein have been deleted and/or wherein one or more amino acids have been inserted in the protein and/or wherein one or more amino acids have been added to the parent protein.

The terms "Factor VII" or "FVII" are intended to include Factor VII polypeptides in their uncleaved (zymogen) form as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated "factor VIIa polypeptides" or "activated factor VII polypeptides").Typically, FVII is cleaved between residues 152 and 153 to yield FVIIa. The term "factor VII polypeptide" is also intended to encompass, without limitation, polypeptides having the amino acid sequence 1-406 of wild-type human Factor VII (as disclosed in U.S. Patent No. 4,784,950), as well as wild-type Factor VII derived from other species, such as, e.g., bovine, porcine, canine, murine, and salmon Factor VII. It further encompasses natural allelic variations of Factor VII that may exist and occur from one individual to another. Also, degree and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells and the nature of the host cellular environment. In one series of embodiments, factor VII polypeptides include polypeptides that exhibit at least about 70 %, preferably at least about 80 %, more preferably at least about 90 %, and most preferable at least about 95 %, of identity with the sequence of wild-type factor VII as disclosed in U.S. Patent No. 4,784,950.

Non-limiting examples of factor VII variants having substantially reduced or modified biological activity relative to wild-type factor VII include R152E-FVIIa (Wildgoose et al., Biochem 29:3413-3420, 1990), S344A-FVIIa (Kazama et al., J. Biol. Chem. 270:66-72, 1995), FFR-FVIIa (Holst et al., Eur. J. Vasc. Endovasc. Surg. 15:515-520, 1998), and factor VIIa lacking the Gla domain, (Nicolaisen et al., FEBS Letts. 317:245-249, 1993). Non-limiting examples also include human FVIIa, which has an amino acid substitution of the lysine corresponding to position 341 of SEQ ID NO: 1; human FVIIa, which has an amino acid substitution of the serine corresponding to position 344 of SEQ ID NO: 1; human FVIIa, which has an amino acid substitution of the aspartic acid corresponding to position 242 of SEQ ID NO: 1; human FVIIa, which has an amino acid substitution of the histidine corresponding to position 193 of SEQ ID NO: 1; FVII-(K341A); FVII-(S344A); FVII-(D242A); and FVII-(H193A). Non-limiting examples of chemically modified factor VII polypeptides and sequence variants are described, e.g., in U.S. Patent No. 5,997,864.

The catalytic activity of Factor VIIa can be inhibited by chemical derivatization of the catalytic center, or triad. Derivatization may be accomplished by reacting Factor VII with an irreversible inhibitor such as an organophosphor compound, a sulfonyl fluoride, a peptide halomethyl ketone or an azapeptide, or by acylation, for example, peptide chloromethylketones or peptidyl cloromethanes; azapeptides; acylating agents such as various guanidinobenzoate derivatives and 3-alkoxy-4-chloroisocoumarins; sulphonyl fluorides such as phenylmethylsulphonylfluoride (PMSF); diisopropylfluorophosphate (DFP); tosylpropylchloromethyl ketone (TPCK); tosylysylchloromethyl ketone (TLCK); nitrophenylsulphonates; heterocyclic protease inhibitors such as isocoumarines, and coumarins.

Preferred peptide halomethyl ketones include Phe-Phe-Arg chloromethyl ketone, Phe-Phe-Arg chloromethylketone, D-Phe-Phe-Arg chloromethyl ketone, D-Phe-Phe-Arg chloromethylketone Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, L-Glu-Gly-Arg chloromethylketone and D-Glu-Gly-Arg chloromethylketone, Dansyl-Phe-Phe-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethylketone, Dansyl-D-Phe-Phe-Arg chloromethyl ketone, Dansyl-D-Phe-Phe-Arg chloromethylketone, Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-D-Phe-Pro-Arg chloromethylketone, Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-D-Phe-Pro-Arg chloromethylketone, Dansyl-L-Glu-Gly-Arg chloromethylketone and Dansyl-D-Glu-Gly-Arg chloromethylketone.

In preferred embodiments amino acid substitutions are made in the amino acid sequence of the Factor VII catalytic triad, defined herein as the regions which contain the amino acids which contribute to the Factor VIIa catalytic site. The substitutions, insertions or deletions in the catalytic triad are generally at or adjacent to the amino acids which form the catalytic site. In the human and bovine Factor VII proteins, the amino acids which form a catalytic "triad" are Ser344, Asp242, and His193 (subscript numbering indicating position in SEQ ID NO:1). The catalytic sites in Factor VII from other mammalian species may be determined using presently available techniques including, among others, protein isolation and amino acid sequence analysis. Catalytic sites may also be determined by aligning a sequence with the sequence of other serine proteases, particularly chymotrypsin, whose active site has been previously determined (Sigler et al., J. Mol. Biol., 35:143-164 (1968), incorporated herein by reference), and therefrom determining from said alignment the analogous active site residues.

The amino acid substitutions, insertions or deletions are made so as to prevent or otherwise inhibit activation by the Factor VIIa of Factors X and/or IX. The Factor VII so modified should, however, also retain the ability to compete with authentic Factor VII and/or Factor VIIa for binding to tissue factor in the coagulation cascade. Such competition may readily be determined by means of, e.g., a clotting assay as described herein, or a competition binding assay using, e.g., a cell line having cell-surface tissue factor, such as the human bladder carcinoma cell line J82 (Sakai et al. J. Biol. Chem. 264: 9980-9988 (1989)).

The amino acids which form the catalytic site in Factor VII, such as Ser344, Asp242, and His193 in human and bovine Factor VII, may either be substituted or deleted. It is preferred to change only a single amino acid, thus minimizing the likelihood of increasing the antigenicity of the molecule or inhibiting its ability to bind tissue factor, however two or more amino acid changes (substitutions, additions or deletions) may be made and combinations of substitution(s), addition(s) and deletion(s) may also be made. In a preferred embodiment for human and bovine Factor VII, Ser344 is preferably substituted with Ala, but Gly, Met, Thr or other amino acids can be substituted. It is preferred to replace Asp with Glu and to replace His with Lys or Arg. In

general, substitutions are chosen to disrupt the tertiary protein structure as little as possible. One may introduce residue alterations as described above in the catalytic site of appropriate Factor VII sequence of human, bovine or other species and test the resulting protein for a desired level of inhibition of catalytic activity and resulting anticoagulant activity as described herein.

In preferred embodiments of human and bovine Factor VII, the active site residue Ser344 is modified, replaced with Gly, Met, Thr, or more preferably, Ala. Such substitution could be made separately or in combination with substitution(s) at other sites in the catalytic triad, which includes His193 and Asp242.

Protein C polypeptides:

In practicing the present invention, any protein C polypeptide may be used that is effective in preventing or treating bleeding. This includes protein C polypeptides derived from blood or plasma, or produced by recombinant means.

The present invention encompasses protein C polypeptides, such as, e.g., those having the amino acid sequence disclosed in Beckmann et al., (Nucleic Acids Research 13:5233 (1985) (wild-type human protein C). In some embodiments, the protein C polypeptide is human activated protein C, as disclosed, e.g., in US 4,981,952. In one series of embodiments, protein C polypeptides include polypeptides that exhibit at least about 10%, preferably at least about 30%, more preferably at least about 50%, and most preferably at least about 70%, of the specific biological activity of human activated protein C. In one series of embodiments, protein C polypeptides include polypeptides that exhibit at least about 90%, preferably at least about 100%, preferably at least about 120%, more preferably at least about 140%, and most preferably at least about 160%, of the specific biological activity of human activated protein C. In one series of embodiments, protein C polypeptides include polypeptides that exhibit at least about 70 %, preferably at least about 80 %, more preferably at least about 90 %, and most preferable at least about 95 %, of identity with the sequence of wild-type protein C as disclosed in Beckmann et al., (Nucleic Acids Research 13:5233 (1985).

As used herein, "protein C polypeptide" encompasses, without limitation, protein C, as well as protein C-related polypeptides. The term "protein C" is intended to encompass, without limitation, polypeptides having the amino acid sequence of wild-type human protein C (as disclosed in Beckmann et al., (Nucleic Acids Research 13:5233 (1985)), as well as wild-type protein C derived from other species, such as, e.g., bovine, porcine, canine, murine, rat and salmon protein C, said protein C derived from blood or plasma, or produced by recombinant means. It further encompasses natural allelic variations of protein C that may exist and occur from one individual to another. Also, degree and location of glycosylation or other post-translation modifications may vary depending on the chosen host cells and the nature of the host cellular environment. The term "protein C" is also intended to encompass protein C polypeptides in their

uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated aPC.

"Protein C-related polypeptides" include, without limitation, protein C polypeptides that have either been chemically modified relative to human protein C and/or contain one or more amino acid sequence alterations relative to human protein C (i.e., protein C variants), and/or contain truncated amino acid sequences relative to human protein C (i.e., protein C fragments). Such protein C-related polypeptides may exhibit different properties relative to human protein C, including stability, phospholipid binding, altered specific activity, and the like. The term "protein C-related polypeptides" are intended to encompass such polypeptides in their uncleaved (zymogen) form, as well as those that have been proteolytically processed to yield their respective bioactive forms, which may be designated "aPC-related polypeptides" or "activated protein C-related polypeptides"

As used herein, "protein C-related polypeptides" encompasses, without limitation, polypeptides exhibiting substantially the same or improved biological activity relative to wild-type human protein C, as well as polypeptides in which the protein C biological activity has been substantially modified or reduced relative to the activity of wild-type human protein C. These polypeptides include, without limitation, protein C or activated protein C that has been chemically modified and protein C variants into which specific amino acid sequence alterations have been introduced that modify or disrupt the bioactivity of the polypeptide.

It further encompasses polypeptides with a slightly modified amino acid sequence, for instance, polypeptides having a modified N-terminal end including N-terminal amino acid deletions or additions, and/or polypeptides that have been chemically modified relative to human protein C.

Protein C-related polypeptides, including variants, whether exhibiting substantially the same or better bioactivity than wild-type protein C, or, alternatively, exhibiting substantially modified or reduced bioactivity relative to wild-type protein C, include, without limitation, polypeptides having an amino acid sequence that differs from the sequence of wild-type protein C by insertion, deletion, or substitution of one or more amino acids.

Protein C-related polypeptides, including variants, encompass those that exhibit at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 100%, at least about 110%, at least about 120%, or at least about 130%, of the specific activity of wild-type activated protein C that has been produced in the same cell type, when tested in the *"Protein C assay"* as described in the present specification.

Protein C-related polypeptides, including variants, having substantially the same or improved biological activity relative to wild-type activated protein C encompass those that

exhibit at least about 25%, preferably at least about 50%, more preferably at least about 75%, more preferably at least about 100%, more preferably at least about 110%, more preferably at least about 120%, and most preferably at least about 130% of the specific activity of wild-type activated protein C that has been produced in the same cell type, when tested in the "*Protein C assay*" as described in the present specification.

Protein C-related polypeptides, including variants, having substantially reduced biological activity relative to wild-type activated protein C are those that exhibit less than about 25%, preferably less than about 10%, more preferably less than about 5% and most preferably less than about 1% of the specific activity of wild-type activated protein C that has been produced in the same cell type when tested in the "*Protein C assay*" as described in the present specification.

In some embodiments the protein C polypeptides are protein C-related polypeptides, in particular variants, wherein the ratio between the activity of said protein C polypeptide and the activity of native human activated protein C (wild-type aPC) is at least about 1.25 when tested in the "*protein C Assay*" as described in the present specification; in other embodiments, the ratio is at least about 2.0; in further embodiments, the ratio is at least about 4.0, in further embodiments, the ratio is at least about 8.0.

In some embodiments, the protein C polypeptide is human protein C, as disclosed, e.g., in Beckmann et al. (see above) (wild-type protein C). In some embodiments, the protein C polypeptide is human activated protein C. In one series of embodiments, the protein C polypeptides are protein C-related polypeptides that exhibits at least about 10%, preferably at least about 30%, more preferably at least about 50%, and most preferably at least about 70%, of the specific biological activity of human activated protein C. In some embodiments, the protein C polypeptides have an amino acid sequence that differs from the sequence of wild-type protein C by insertion, deletion, or substitution of one or more amino acids.

In the present context the three-letter or one-letter indications of the amino acids have been used in their conventional meaning as indicated in table 1. Unless indicated explicitly, the amino acids mentioned herein are L-amino acids. It is to be understood, that the first letter in, for example, K337 represent the amino acid naturally present at the indicated position wild-type factor VII, and that, for example, [K337A]-FVIIa designates the FVII-variant wherein the amino acid represented by the one-letter code K naturally present in the indicated position is replaced by the amino acid represented by the one-letter code A.

Table 1: Abbreviations for amino acids:

Amino acid	Tree-letter code	One-letter code
Glycine	Gly	G
Proline	Pro	P

Alanine	Ala	A
Valine	Val	V
Leucine	Leu	L
Isoleucine	Ile	I
Methionine	Met	M
Cysteine	Cys	C
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Tryptophan	Trp	W
Histidine	His	H
Lysine	Lys	K
Arginine	Arg	R
Glutamine	Gln	Q
Asparagine	Asn	N
Glutamic Acid	Glu	E
Aspartic Acid	Asp	D

The term "factor VIIa" or "FVIIa" may be used interchangeably.

The term "active site" and the like when used herein with reference to FVIIa refer to the catalytic and zymogen substrate binding site, including the "S1" site of FVIIa as that term is defined by Schecter, I. and Berger, A., (1967) Biochem. Biophys. Res. Commun. 7:157- 162.

The term "TF-mediated coagulation activity" means coagulation initiated by TF through the formation of the TF/FVIIa complex and its activation of FIX and Factor X to FIXa and FXa, respectively. TF-mediated coagulation activity is measured in a FXa generation assay. The term "FXa generation assay" as used herein is intended to mean any assay where activation of FX is measured in a sample comprising TF, FVIIa, FX, calcium and phospholipids. Examples of FXa generation assays are described in assay 1 and assay 2 (of the present specification).

A TF/FVIIa mediated or associated process or event, or a process or event associated with TF-mediated coagulation activity, is any event, which requires the presence of TF/FVIIa.

Such processes or events include, but are not limited to, formation of fibrin which leads to thrombus formation; platelet deposition; proliferation of smooth muscle cells (SMCs) in the vessel wall, such as, for example, in intimal hyperplasia or restenosis, which is thought to result from a complex interaction of biological processes including platelet deposition and thrombus formation, release of chemotactic and mitogenic factors, and the migration and proliferation of vascular smooth muscle cells into the intima of an arterial segment; and deleterious events associated with post-ischemic reperfusion, such as, for example, in patients with acute myocardial infarction undergoing coronary thrombolysis.

The general mechanism of blood clot formation is reviewed by Ganong, in Review of Medical Physiology, 13th ed., Lange, Los Altos Calif., pp 411-414 (1987). Coagulation requires the confluence of two processes, the production of thrombin which induces platelet aggregation and the formation of fribrin which renders the platelet plug stable. The process comprises sev-

eral stages each requiring the presence of discrete proenzymes and profactors. The process ends in fibrin crosslinking and thrombus formation. Fibrinogen is converted to fibrin by the action of thrombin. Thrombin, in turn, is formed by the proteolytic cleavage of prothrombin. This proteolysis is effected by factor Xa which binds to the surface of activated platelets and in the presence of FVa and calcium, cleaves prothrombin. TF/FVIIa is required for the proteolytic activation of factor X by the extrinsic pathway of coagulation. Therefore, a process mediated by or associated with TF/FVIIa, or an TF-mediated coagulation activity includes any step in the coagulation cascade from the formation of the TF/FVIIa complex to the formation of a fibrin platelet clot and which initially requires the presence of TF/FVIIa. For example, the TF/FVIIa complex initiates the extrinsic pathway by activation of factor X to factor Xa, FIX to FIXa, and additional FVII to FVIIa. TF/FVIIa mediated or associated process, or TF-mediated coagulation activity can be conveniently measured employing standard assays such as those described in Roy, S., (1991) J. Biol. Chem. 266:4665-4668, and O'Brien, D. et al., (1988) J. Clin. Invest. 82:206-212 for the conversion of factor X to factor Xa in the presence of TF/FVIIa and other necessary reagents.

The term "TF related diseases or disorders" as used herein means any disease or disorder, where TF is involved. This includes, but are not limited to diseases or disorders related to TF-mediated coagulation activity, thrombotic or coagulopathic related diseases or disorders or diseases or disorders such as inflammatory responses and chronic thromboembolic diseases or disorders associated with fibrin formation, including vascular disorders such as deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, tumour metastasis, angiogenesis, thrombolysis, arteriosclerosis and restenosis following angioplastry, acute and chronic indications such as inflammation, septic chock, septicemia, hypotension, adult respiratory distress syndrome (ARDS), disseminated intravascular coagulopathy (DIC), pulmonary embolism, platelet deposition, myocardial infarction, or the prophylactic treatment of mammals with atherosclerotic vessels at risk for thrombosis, and other diseases. The TF related diseases or disorders are not limited to in vivo coagulopatic disorders such as those named above, but includes ex vivo TF/FVIIa related processes such as coagulation that may result from the extracorporeal circulation of blood, including blood removed in-line from a patient in such processes as dialysis procedures, blood filtration, or blood bypass during surgery.

It should be noted that peptides, proteins and amino acids as used herein can comprise or refer to "natural", i.e., naturally occurring amino acids as well as "non.classical" D-amino acids including, but not limited to, the D-isomers of the common amino acids, α-isobutyric acid, 4-aminobutyric acid, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general. In addition, the amino acids can include Abu, 2-amino butyric acid; γ-Abu, 4-aminobutyric

acid; ε-Ahx, 6-aminohexanoic acid; Aib, 2-amino-isobutyric acid; β-Ala, 3-aminopropionic acid; Orn, ornithine; Hyp, trans-hydroxyproline; Nle, norleucine; Nva, norvaline.

The three-letter indication "GLA" as used herein means 4-carboxyglutamic acid (γ-carboxyglutamate).

The terms **"human tissue factor"** or **"human TF"** as used herein, refers to the full length polypeptide receptor comprising the amino acid sequence 1-263 of native human tissue factor.

The term **"antibody"**, as used herein, is intended to refer to immunoglobulin molecules and fragments thereof, which have the ability to specifically bind to an antigen (e.g., human TF). Full-length antibodies comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. Thus, within the definition of an antibody is also one or more fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., human TF). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antibody" include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH I domains; (ii) F(ab)2 and F(ab')2 fragments, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242:423-426: and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antibody". Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary

domains of another chain and creating two antigen binding sites (see e.g., Holliger, P., et al. (1993) Proc Natl. Acad. Sci. USA 90:6444-6448; Poljak, R. J., et al. (1994) Structure 2:1121-1123). It is understood that human TF may have one or more antigenic determinants comprising (1) peptide antigenic determinants which consist of single peptide chains within human TF, (2) conformational antigenic determinants which consist of more than one spatially contiguous peptide chains whose respective amino acid sequences are located disjointedly along the human TF polypeptide sequence; and (3) post-translational antigenic determinants which consist, either in whole or part, of molecular structures covalently attached to human TF after translation, such as carbohydrate groups, or the like.

The terms "**human antibody**", "human antibodies", "human TF antibody", and "human TF antibodies", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), for example in the CDRs and in particular CDR3. However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences, e.g. the so-called humanized antibodies or human/mouse chimera antibodies.

By "**catalytically inactivated in the active site of the FVIIa polypeptide**" is meant that a factor VIIa inhibitor is bound to the factor VIIa polypeptide and decreases or prevents the factor VIIa-catalysed conversion of factor X to factor Xa. A FVIIa inhibitor may be identified as a substance, which reduces the amidolytic activity by at least 50% at a concentration of the substance at 400 μM in the FVIIa amidolytic assay described by Persson et al. (Persson et al., J. Biol. Chem. 272: 19919-19924 (1997)). Preferred are substances reducing the amidolytic activity by at least 50% at a concentration of the substance at 300 μM; more preferred are substances reducing the amidolytic activity by at least 50% at a concentration of the substance at 200 μM.

The "**FVIIa inhibitor**" may be selected from any one of several groups of FVIIa directed inhibitors. Such inhibitors are broadly categorised for the purpose of the present invention into i) inhibitors which reversibly bind to FVIIa and are cleavable by FVIIa, ii) inhibitors which reversibly bind to FVIIa but cannot be cleaved, and iii) inhibitors which irreversibly bind to FVIIa. For a review of inhibitors of serine proteases see Proteinase Inhibitors (Research Monographs in cell and Tissue Physiology; v. 12) Elsevier Science Publishing Co., Inc., New York (1990).

The FVIIa inhibitor moiety may also be an irreversible FVIIa serine protease inhibitor. Such irreversible active site inhibitors generally form covalent bonds with the protease active site Such irreversible inhibitors include, but are not limited to, general serine protease inhibitors such as peptide chloromethylketones (see, Williams et al., J. Biol. Chem. 264:7536-7540 (1989)) or

peptidyl cloromethanes; azapeptides; acylating agents such as various guanidinobenzoate derivatives and the 3-alkoxy-4-chloroisocoumarins; sulphonyl fluorides such as phenylmethylsulphonylfluoride (PMSF); diisopropylfluorophosphate (DFP); tosylpropylchloromethyl ketone (TPCK); tosyllysylchloromethyl ketone (TLCK); nitrophenylsulphonates and related compounds; heterocyclic protease inhibitors such as isocoumarines, and coumarins.

Examples of peptidic irreversible FVIIa inhibitors include, but are not limited to, Phe-Phe-Arg chloromethyl ketone, Phe-Phe-Arg chloromethylketone, D-Phe-Phe-Arg chloromethyl ketone, D-Phe-Phe-Arg chloromethylketone Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, L-Glu-Gly-Arg chloromethylketone and D-Glu-Gly-Arg chloromethylketone.

Examples of FVIIa inhibitors also include benzoxazinones or heterocyclic analogues thereof such as described in PCT/DK99/00138.

Examples of other FVIIa inhibitors include, but are not limited to, small peptides such as for example Phe-Phe-Arg, D-Phe-Phe-Arg, Phe-Phe-Arg, D-Phe-Phe-Arg, Phe-Pro-Arg, D-Phe-Pro-Arg, Phe-Pro-Arg, D-Phe-Pro-Arg, L- and D-Glu-Gly-Arg; peptidomimetics; benzamidine systems; heterocyclic structures substituted with one or more amidino groups; aromatic or heteroaromatic systems substituted with one or more C(=NH)NHR groups in which R is H, C1-3alkyl, OH or a group which is easily split of *in vivo*.

The terms "aPC", **"Activated human Protein C"**, "Activated Protein C", "raPC", and "recombinant Activated Protein C" are synonymous for the purpose and practice of this invention and can be used interchangeably.

Protein C Activity: any property of activated human Protein C or its derivatives responsible for proteolytic, amidolytic, esterolytic, and biological (anticoagulant or pro-fibrinolytic) activities. Methods for testing for Protein C anticoagulant and amidolytic activity are well known in the art, i.e., see Grinnell et.al., 1987, Bio/Technology 5:1189-1192.

RhaPC: Recombinant activated human protein C, produced by activating r-HPC in vitro or by direct secretion of the activated form of Protein C from prokaryotic cells, eukaryotic cells, or from transgenic animals.

Zymogen: an enzymatically inactive precursor of a proteolytic enzyme. Protein C zymogen, as used herein, refers to secreted, inactive forms, whether one chain or two chain, of protein C.

Thrombotic or coagulopathic related diseases or disorders: the term includes vascular diseases and inflammatory responses including, without limitation, deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, tumour metastasis, inflammation, septic chock, hypotension, acute lung injury (ALI), Acute Respiratory Distress Syndromr (ARDS), pulmonary embolism, disseminated intravascular coagulation (DIC), sepsis, systemic inflammatory response

syndrome (SIRS), vascular restenosis, platelet deposition, myocardial infarction, angiogenesis, or the treatment of mammals with atherosclerotic vessels at risk for thrombosis, and multiple organ failure resulting from any of the preceding pathologic processes.

Respiratory Diseases or disorders: exemplified by lower respiratory diseases such as systemic inflammatory response syndrome, asthma, bronchitis, acute lung injury, acute resporatory distress syndrome, idiopathic pulmonary fibrosis, pneumonia, pulmonary edema, pulmonary obstructive disease, endotoxin induced lung damage, non cell lung cancer, and multiple organ failure resulting from any of the preceding pathologic processes.

Inflammatory Diseases or disorders: refers to diseases such as inflammatory bowel disease, sepsis, septic shock, acute respiratory distress syndrome, pancreatitis, trauma-induced shock, bronchial asthma, allergic rhinitis, rheumatoid arthritis, cystic fibrosis, stroke, acute bronchitis, chronic bronchitis, acute bronchiolitis, chronic bronchiolitis, osteoarthritis, gout, spondylarthropathris, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, enterapathric spondylitis, juvenile arthropathy or juvenile ankylosing spondylitis, reactive arthropathy, infectious or post-infectious arthritis, gonoccocal arthritis, tuberculous arthritis, viral arthritis, fungal arthritis, syphilitic arthritis, Lyme disease, arthritis associated with "vasculitic syndromes," polyarteritis nodosa, hypersensitivity vasculitis, Luegenec's granulomatosis, polymyalgin rheumatica, joint cell arteritis, calcium crystal deposition arthropathris, pseudo gout, non-articular rheumatism, bursitis, tenosynomitis, epicondylitis (tennis elbow), carpal tunnel syndrome, repetitive use injury (typing), miscellaneous forms of arthritis, neuropathic joint disease (charco and joint), hemarthrosis (hemarthrosic), Henoch-Schonlein Purpura, hypertrophic osteoarthropathy, multicentric reticulohistiocytosis, arthritis associated with certain diseases, surcoilosis, hemochromatosis, sickle cell disease and other hemoglobinopathries, hyperlipoproteineimia, hypogammaglobulinemia, hyperparathyroidism, acromegaly, familial Mediterranean fever, Behat's Disease, systemic lupus erythrematosis, relapsing, and multiple organ failure resulting from any of the preceding pathologic processes.

The phrase **"therapeutically effective interval"** is a period of time beginning when one of either (a) the TF antagonist or (b) Protein C or protein C-related polypeptide is administered to a mammal and ending at the limit of the beneficial effect in preventing or ameliorating respiratory or inflammatory disease or associated organ failure of (a) or (b).

"Sole" agents or factors as used herein refers to situations in which the TF antagonist and the protein C or protein C-related polypeptide, taken together, are the only haemostatic agents, or active haemostatic agents, or coagulation factors contained in the pharmaceutical composition or kit, or are the only haemostatic agents,or active haemostatic agents, or coagulation factors administered to the patient in the course of a particular treatment, such as, e.g., in the course of a particular bleeding episode. It will be understood that these situations encompass those in which other haemostatic agents or coagulation factors, as applicable, are not pre-

sent in either sufficient quantity or activity so as to significantly influence one or more coagulation parameters.

Clot lysis time, clot strength, fibrin clot formation, and clotting time are clinical parameters used for assaying the status of patient's haemostatic system. Blood samples are drawn from the patient at suitable intervals and one or more of the parameters are assayed by means of, e.g., thromboelastograpy as described by, e.g., Meh et al.,Blood Coagulation & Fibrinolysis 2001;12:627-637; Vig et al., Hematology, Vol. 6 (3) pp. 205-213 (2001); Vig et al., Blood coagulation & fibrinolysis, Vol. 12 (7) pp 555-561 (2001) Oct; Glidden et al., Clinical and applied thrombosis/hemostasis, Vol. 6 (4) pp. 226-233 (2000) Oct; McKenzie et al., Cardiology, Vol. 92 (4) pp. 240-247 (1999) Apr; or Davis et al., Journal of the American Society of Nephrology, Vol. 6 (4) pp. 1250-1255 (1995).

In this context, the term **"treatment"** is meant to include both prevention of an expected unwanted clotting, and regulation of an already occurring clotting. Prophylactic administration of a preparation of a TF antagonist and a protein C polypeptide is thus included in the term "treatment".

The term **"subject"** as used herein is intended to mean any animal, in particular mammals, such as humans, and may, where appropriate, be used interchangeably with the term "patient".

The TF antagonist and protein C or protein C-related polypeptide as defined in the present specification may be administered simultaneously or sequentially. The factors may be supplied in single-dosage form wherein the single-dosage form contains both coagulation factors, or in the form of a kit-of-parts comprising a preparation of a TF antagonist as a first unit dosage form and a preparation of protein C or protein C-related polypeptide as a second unit dosage form. Whenever a first or second or third, etc., unit dose is mentioned throughout this specification this does not indicate the preferred order of administration, but is merely done for convenience purposes

By **"simultaneous"** dosing of a preparation of a TF antagonist and a preparation of protein C or protein C-related polypeptide is meant administration of the coagulation factor proteins in single-dosage form, or administration of a first coagulation factor protein followed by administration of a second coagulation factor protein with a time separation of no more than 15 minutes, preferably 10, more preferred 5, more preferred 2 minutes. Either factor may be administered first.

By **"sequential"** dosing is meant administration of a first coagulation factor protein followed by administration of a second coagulation factor protein with a time separation of more than 15 minutes. Either of the two unit dosage form, or coagulation factor proteins, may be administered first. Preferably, both products are injected through the same intravenous access.

By **"APTT"** or "aPTT" is meant the activated partial thromboplastin time (described by, e.g., Proctor RR, Rapaport SI: The partial thromboplastin time with kaolin; a simple screening test for first-stage plasma clotting factor deficiencies. Am J Clin Pathol 36:212, 1961).

"**Half-life**" refers to the time required for the plasma concentration of a TF antagonist or protein C or a protein C-related polypeptide to decrease from a particular value to half of that value.

The total amount of protein in a preparation may be measured by generally known methods, e.g, by measuring optical density. Amounts of protein C polypeptides or factor VII protein ("antigen") may be measured by generally known methods such as standard Elisa immuno assays. In general terms, such assay is conducted by contacting, e.g., a solution of the protein C-containing preparation with an anti-protein C antibody immobilised onto the elisa plate, subsequently contacting the immobilised antibody-protein C complex with a second anti protein C antibody carrying a marker, the amounts of which, in a third step, are measured. The amounts of each coagulation factor may be measured in a similar way using appropriate antibodies. The total amount of coagulation factor protein present in a preparation is determined by adding the amounts of the individual coagulation factor proteins. In one embodiment, the preparation comprises isolated coagulation factor. In another embodiment the preparation is free of coagulation factor II and coagulation factor IIa (prothrombin and thrombin) and/or factor X or Xa.

As used herein, the term "**isolated**" refers to coagulation factors, e.g., protein C or protein C-related polypeptides that have been separated from the cell in which they were synthesized or the medium in which they are found in nature (e.g., plasma or blood). Separation of polypeptides from their cell of origin may be achieved by any method known in the art, including, without limitation, removal of cell culture medium containing the desired product from an adherent cell culture; centrifugation or filtration to remove non-adherent cells; and the like. Separation of polypeptides from the medium in which they naturally occur may be achieved by any method known in the art, including, without limitation, affinity chromatography, such as, e.g., on an anti-factor VII or anti-protein C antibody column, respectively; hydrophobic interaction chromatography; ion-exchange chromatography; size exclusion chromatography; electrophoretic procedures (e.g., preparative isoelectric focusing (IEF)), differential solubility (e.g., ammonium sulfate precipitation), or extraction and the like.

Within the present invention an "**effective amount**" of a TF antagonist and a protein C or protein C-related polypeptide is defined as the amount of a TF antagonist, e.g., inactivated FVIIa or a TF antibody, and a protein C polypeptide that together suffices to cure, alleviate or partially arrest the disease and its complications.

The phrase "**therapeutically effective combination,**" used in the practice of this invention, means administration of both (a) a TF antagonist and (b) protein C or a protein C-related polypeptide, either simultaneously or separately.

The term, "**Active Ingredient**" as used herein refers to a combination of (a) a TF antagonist and (b) Protein C or a protein C-related polypeptide co-present in a pharmaceutical formulation for the delivery of a treatment regimen that applies this invention.

The term, **"injectable liquid carrier"** refers to a liquid medium containing either or both of (a) a TF antagonist, or (b) Protein C or a protein C-related polypeptide; wherein (a) and (b) are independently dissolved, suspended, dispersed, or emulsified in the liquid medium.

Abbreviations

TF	tissue factor
FVII	factor VII in its single-chain, unactivated form
FVIIa	factor VII in its activated form
rFVIIa	recombinant factor VII in its activated form
APC	Activated human Protein C, also called, Activated Protein C.
HPC	human Protein C zymogen.
rhPC	recombinant human Protein C zymogen.
APTT	activated partial thromboplastin time.

Preparation of compounds:

Methods for preparing recombinant proteins including conventional molecular biology, microbiology, and recombinant DNA techniques are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989") DNA Cloning: A Practical Approach, Volumes I and II /D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984).

Briefly, DNA sequences encoding a specific protein (e.g., protein C or human FVIIa) may be isolated by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the protein by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., above). For the present purpose, the DNA sequence encoding the protein is preferably of human origin, i.e. derived from a human genomic DNA or cDNA library.

Polypeptide variants may be made by amino acid sequence alterations of the polypeptide, which may be accomplished by a variety of techniques. Modification of the DNA sequence may be by site-specific mutagenesis. Techniques for site-specific mutagenesis are well known in the art and are described by, for example, Zoller and Smith (DNA 3:479-488, 1984).

The DNA sequences encoding the polypeptide may also be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by Beaucage and

Caruthers, Tetrahedron Letters 22 (1981), 1859 - 1869, or the method described by Matthes et al., EMBO Journal 3 (1984), 801 - 805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA sequences may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202, Saiki et al., Science 239 (1988), 487 - 491, or Sambrook et al., supra.

The host cell into which the DNA sequences encoding the polypeptides is introduced may be any cell, which is capable of producing the posttranslational modified human FVIIa polypeptides and includes yeast, fungi and higher eucaryotic cells. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845.

Methods for producing antibodies are generally known in the art, see, e.g., Harboe and Ingild, In N.H. Axelsen, J. Krøll, and B. Weeks, editors, A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or Johnstone and Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, 1982 (more specifically pages 27-31). Preferably, the antibodies are monoclonal antibodies. Monoclonal antibodies may be prepared, e.g., according to the methods of E. Harlow and D. Lane, editors, 1988, Antibodies, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York.

Methods of preparing human antibodies against human TF is, for example, described in International application No. PCT/DK02/00644

Factor VII polypeptides

For the preparation of recombinant human FVIIa polypeptides, a cloned wild-type FVIIa DNA sequence is used. This sequence may be modified to encode a desired FVIIa variant. The complete nucleotide and amino acid sequences for human FVIIa are known; see U.S. Pat. No. 4,784,950, where the cloning and expression of recombinant human FVIIa is described. The bovine FVIIa sequence is described in Takeya et al., J. Biol. Chem, 263:14868-14872 (1988), which is incorporated by reference herein.

DNA sequences for use will typically encode a pre-pro peptide at the amino-terminus of the FVIIa protein to obtain proper post-translational processing (e.g. gamma-carboxylation of glutamic acid residues) and secretion from the host cell. The pre-pro peptide may be that of FVIIa or another vitamin K-dependent plasma protein, such as factor IX, factor X, prothrombin, protein C or protein S. As will be appreciated by those skilled in the art, additional modifications can

be made in the amino acid sequence of FVIIa where those modifications do not significantly have impact on the ability of the protein to act as a coagulation factor. For example, FVIIa in the catalytic triad can also be modified in the activation cleavage site to inhibit the conversion of zymogen FVII into its activated two-chain form, as generally described in U.S. Pat. No. 5,288,629.

Factor VII polypeptides for use in the present invention may be prepared, e.g., as described in International Applications Nos. WO 92/15686, WO 94/27631 and WO 96/12800; Wildgoose et al., Biochem 29:3413-3420, 1990; Kazama et al., J. Biol. Chem. 270:66-72, 1995; Holst et al., Eur. J. Vasc. Endovasc. Surg. 15:515-520, 1998; and Nicolaisen et al., FEBS Letts. 317:245-249, 1993.

FVII polypeptides produced as described above may be purified by affinity chromatography on an anti-FVII antibody column. The immunoadsorption column comprise a high-specificity monoclonal antibody, such as, e.g., a calcium-dependent monoclonal antibody as described by Wakabayashi et al., J. Biol. Chem, 261:11097-11108, (1986) and Thim et al., Biochem. 27: 7785-7793, (1988). Additional purification may be achieved by conventional chemical purification means, such as high performance liquid chromatography. Other methods of purification, including barium citrate precipitation, are known in the art, and may be applied to the purification of the FVIIa described herein (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y., 1982).

Conversion of single-chain FVII to active two-chain FVIIa may be achieved using factor XIIa as described by Hedner and Kisiel (1983, J. Clin. Invest. 71: 1836-1841), or using other proteases having trypsin-like specificity (Kisiel and Fujikawa, Behring Inst. Mitt. 73: 29-42, 1983). Alternatively FVII may be autoactivated by passing it through an ion-exchange chromatography column, such as mono Q.RTM. (Pharmacia Fire Chemicals) or the like (Bjoern et al., 1986, Research Disclosures 269:564-565).

Preparation of the Activated Protein C Ingredient.

The cloning oh human protein C has been described by Beckmann et al., (Nucleic Acids Research 13:5233 (1985) (wild-type human protein C). The expression of recombinant human Protein C in human kidney 293 cells has been described by Grinnell et al. (BioTechnology 5:1189-1192 (1987)). Recombinant human Protein C (r-hPC) may be produced by techniques well known to the skilled artisan such as those set forth in Yan, U.S. Patent No. 4,981,952. The gene encoding human Protein C is disclosed and claimed in Bang et al., U.S. Patent No. 4,775,624. A plasmid useful to express human Protein C in 293 cells (pLPC) is disclosed in Bang et al., U.S. Patent No. 4,992,373; the construction of plasmid pLPC is also described in European Patent Publication No. 0 445 939 and in Grinnell et al., 1987, Bio/Technology 5:1189-1192. Briefly, the plasmid was transfected into 293 cells; stable transformants were identified, subcultured and grown in serum-free media. After fermentation, cell-free medium was obtained by microfiltration.

Production and isolation of Protein C is also described by Haley et al., J. Biol. Chem., 264; 16303, 1989, and Turkay et al., Thromb. Haemost. 81; 727 1999.

Pharmaceutical Compositions and Methods of Use

The preparations of the present invention may be used to treat thrombotic and coagulopathic related diseases or disorders, respiratory diseases or disorders, and inflammatory diseases or disorders including, without limitation, deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, tumour metastasis, inflammation, septic chock, hypotension, acute lung injury (ALI), Acute Respiratory Distress Syndromr (ARDS), pulmonary embolism, disseminated intravascular coagulation (DIC), sepsis, systemic inflammatory response syndrome (SIRS), vascular restenosis, platelet deposition, myocardial infarction, angiogenesis, or the prophylactic treatment of mammals with atherosclerotic vessels at risk for thrombosis; asthma, bronchitis, idiopathic pulmonary fibrosis, pneumonia, pulmonary edema, pulmonary obstructive disease, endotoxin induced lung damage, non cell lung cancer; inflammatory bowel disease, pancreatitis, trauma-induced shock, bronchial asthma, allergic rhinitis, rheumatoid arthritis, cystic fibrosis, stroke, acute bronchitis, chronic bronchitis, acute bronchiolitis, chronic bronchiolitis, osteoarthritis, gout, spondylarthropathris, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, enterapathric spondylitis, juvenile arthropathy or juvenile ankylosing spondylitis, reactive arthropathy, infectious or post-infectious arthritis, gonoccocal arthritis, tuberculous arthritis, viral arthritis, fungal arthritis, syphilitic arthritis, Lyme disease, arthritis associated with "vasculitic syndromes," polyarteritis nodosa, hypersensitivity vasculitis, Luegenec's granulomatosis, polymyalgin rheumatica, joint cell arteritis, calcium crystal deposition arthropathris, pseudo gout, non-articular rheumatism, bursitis, tenosynomitis, epicondylitis (tennis elbow), carpal tunnel syndrome, repetitive use injury (typing), miscellaneous forms of arthritis, neuropathic joint disease (charco and joint), hemarthrosis (hemarthrosic), Henoch-Schonlein Purpura, hypertrophic osteoarthropathy, multicentric reticulohistiocytosis, arthritis associated with certain diseases, surcoilosis, hemochromatosis, sickle cell disease and other hemoglobinopathries, hyperlipoproteineimia, hypogammaglobulinemia, hyperparathyroidism, acromegaly, familial Mediterranean fever, Behat's Disease, systemic lupus erythrematosis, relapsing, and multiple organ failure resulting from any of the preceding pathologic processes.

The essential ingredients (a) a TF antagonist and (b) Protein C or a protein C-related polypeptide are present in the formulation in such proportion that a dose of the formulation provides an amount of each ingredient that together is a pharmaceutically effective amount to the patient being treated.The dose of composition of the invention to be administered is determi-

ned depending upon age, body weight, symptom, the desired therapeutic effect, the route of administration, and the duration of the treatment etc. Typically, the weight ratio of TF antagonist and the amount of protein C or protein C-related polypeptide (e.g., APC or a biologically active fragment or variant thereof) may vary from a ratio of between about 1:100 to about 100:1 (w/w). The ratio of TF antagonist to protein C or protein C-related polypeptide (e.g., APC or biologically active fragment or variant) may thus be, e.g., about 1:100, or 1:90, or 1:80, or 1:70 or 1:60, or 1:50, or 1:40, or 1:30, or 1:20, or 1:10, or 1:5, or 1:2, or 1:1, or 2:1, or 5:1, or 10:1, or 20:1, or 30.1, or 40:1, or 50:1, or 60:1, or 70:1, or 80:1, or 90:1, or 100:1; or between about 1:90 to about 1:1, or between about 1:80 to about 1:2, or between about 1:70 to about 1:5, or between about 1:60 to about 1:10, or between about 1:50 to about 1:25, or between about 1:40 to about 1:30, or between about 90:1 to about 1:1, or between about 80:1 to about 2:1, or between about 70:1 to about 5:1, or between about 60:1 to about 10:1, or between about 50:1 to about 25:1, or between about 40:1 to about 30:1; or between about 10:1 to about 1:10, or between about 5:1 to about 1:5.

The dose of the TF antagonist ranges from about 0.05 mg to about 500 mg/day, e.g., from about 1 mg to about 200 mg/day, or, e.g., from about 5 mg to about 175 mg/day for a 70-kg subject as loading and maintenance doses, depending on the weight of the subject, the condition and the severity of the condition.

The dose of the protein C or protein C-related polypeptide ranges from about 0.05 mg to about 500 mg/day, e.g., from about 1 mg to about 200 mg/day, or, e.g., from about 5 mg to about 175 mg/day for a 70-kg subject as loading and maintenance doses, depending on the weight of the subject, the condition and the severity of the condition.

It must be kept in mind that the materials of the present invention may generally be employed in serious disease or injury states, that is, life threatening or potentially life threatening situations. In such cases, in view of the minimization of extraneous substances and general lack of immunogenicity of factor VIIa and protein C in humans, it is possible and may be felt desirable by the treating physician to administer a substantial excess of these compositions.

A dose may be given continuously or intermittently (once or several times a day). A course of treatment is typically from I to 30 days. In making compositions of the invention the essential ingredients; TF antagonist and Protein C are co-present and may be mixed in any homogeneous or non-homogeneous manner or adjacently or otherwise promixately placed together in an individual dosage unit suitable for practicing the method of the invention.The dosage unit of the TF antagonist will usually be admixed with a carrier or inert ingredients, or diluted by a carrier, or enclosed within a carrier which may be in the form of a ampoule, capsule, time release dosing device, sachet, paper or other container. When the carrier serves as a diluent, it may be a solid, semi-solid, paste, or liquid material which acts as a vehicle, or can be in the form of tablets, pills, powders, lozenges, elixirs, suspensions, emulsions, solutions, syrups, aero-

sols (as a solid or in a liquid medium), or ointment, containing, for example, up to 10% by weight of the active compound.The dosage unit of the Protein C polypeptide will usually be admixed with a liquid carrier and/or other inert ingredients or enclosed within a carrier which may be in the form of a ampoule, bottle, time release dosing device or other container. When the carrier serves as a diluent, it may be a liquid material which acts as a vehicle, or can be in the form of solutions containing, for example, up to 10% by weight of the active compound.The Protein C ingredient should be in an injectable liquid form immediately prior to use, however, it may be made in a storable form which is not a liquid but is easily convertable to a liquid (e.g., paste, liquid adsorbed on a solid, etc.) For the pharmaceutical formulations containing both (a) TF antagonist and (b) Protein C the carrier may be an injectable liquid medium such as is well known in the art. The injectable liquid must be such that permits parenteral administration, that is, introduction of substances to a mammal being treated by intervenous, subcutaneous, intramuscular, or intramedullary injection. Intravenous injection is most preferred as a means of administration.The Active ingredient can be dissolved or suspended in a pharmaceutically acceptable carrier, such as sterile water, sterile water containing saline and/or sugars and/or suspension agents or a mixture of both. For example, for intravenous injection the compounds of the invention may be dissolved in at a concentration of about 2 mg/ml in a 4% dextrose/0.5% Na citrate aqueous solution. Liquid compositions for oral administration include pharmaceutically-acceptable emulsions, solutions, suspensions, syrups and elixirs containing inert diluents commonly used in the art such as distilled water or ethanol. Besides inert diluents such compositions may also comprise adjuvants such as wetting and suspending agents, and sweetening, flavouring, perfuming and preserving agents. Other compositions for oral administration include spray compositions which may be prepared by known methods and which comprise one or more of the active compound(s). Besides inert diluents such compositions may also comprise stabilizers such as sodium bisulfite and buffer for isotonicity, for example sodium chloride, sodium citrate or citric acid.The manufacturing methods of spray compositions for inhalation therapy is described in detail, for example, in the specifications of U.S. Pat. No. 2,868,691 and U.S.Pat. No. 3,095,355. Preparations for injection according to the present invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions or emulsions. Example of aqueous solvents or suspending media are distilled water for injection and physiological salt solution. Examples of non-aqueous solvents or sus-oendincf media are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, alcohols such as ehtanol, Polysorbate 80 (registered Trade Mark). These compositions may also include adjuvants such as preserving, wetting, emulsifying and dispersing agents stabilizing agents (e.g. lactose) and solubilizers (e.g. glutamic acid and asparaginic acid).They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporation of sterilizing agents in the compositions or by irradiation. They may also be manufactured in the form of sterile solid compositions which can be dissolved in sterile water

or some other sterile injectable medium immediately before use. The TF antagonist (when separate from the Protein C polypeptide) may be in the form of powder, tablet or capsule. A solid carrier can be one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, binders, tablet disintegrating agents and encapsulating material. Suitable solid carriers are magnesium carbonate, magnesium stearate, talc, sugar lactose, pectin, dextrin, starch, gelatin, tragacanth, methyl cellulose, sodium carboxymethyl cellulose, low melting waxes, and cocoa butter. Preferably, the pharmaceutical compositions are administered parenterally, i.e., intravenously, subcutaneously, or intramuscularly; intravenously being most preferred. They may also be administered by continuous or pulsatile infusion. Local delivery of the preparations of the present invention, such as, for example, topical application, may be carried out, e.g., by means of a spray, perfusion, double balloon catheters, stent, incorporated into vascular grafts or stents, hydrogels used to coat balloon catheters, or other well established methods. One skilled in this art may formulate the compositions of the invention an appropriate manner, and in accordance with accepted practices, such as those disclosed in Remington's Pharmaceutical Sciences, Gennaro, ed., Mack Publishing Co., Easton, PA, 1990.

This invention is a method of treating or preventing thrombotic and coagulopathic diseases, Inflammatory Disease or Respiratory Disease by administering to a mammal in need thereof a therapeutically effective amount of (a) a TF antagonist and b) a Protein C polypeptide; wherein (a) and (b) are both administered within a therapeutically effective interval. The administration of (a) or (b) to, e.g., a septic patient may be either continuous or intermittent.

The Protein C polypeptide and a TF antagonist can be delivered simultaneously. One convenient method of simultaneous delivery is to use the compositions of the invention, wherein the Active Ingredient has the essential ingredients co-present in a unit dosage form. Solutions or suspensions of mixed essential ingredients may, if desired, be delivered from the same liquid holding bag. Another method of simultaneous delivery of the Protein C polypeptide and a TF antagonist is to deliver them to the patient separately but simultaneously. Thus, for example, some TF antagonists may be given as an oral formulation at the same time as the Protein C polypeptide is given parenterally. Dosage of a TF antagonist can begin simultaneously with the Protein C administration. The length of the TF antagonist administration can extend past the Protein C administration, or vice versa.

Each of the essential ingredients, viz., a therapeutically effective amount of (a) a TF antagonist in and (b) Protein C polypeptide have a therapeutically effective interval, namely the interval of time in which each agent provides benefit for the patient being treated with Thrombotic or Coagulopathic related disease, Inflammatory Disease or Respiratory Disease. The method of the invention may be practiced by separately dosing the patient in any order with a therapeu-

tically effective amount of (a) a TF antagonist and (b) Protein C polypeptide provided that each agent is given within the period of time that that the other agent is therapeutically effective against Thrombotic or Coagulopathic related disease, Inflammatory Disease or Respiratory Disease, or organ failure resulting from these pathologic processes.

The Protein C polypeptide and TF antagonist are preferably administered parenterally to a patient to insure their delivery into the bloodstream in an effective form as fast as possible.

The amount and relative ratio of protein C polypeptide and TF antagonist to be used in the practice of the method of invention is set out in the previous section. It may be appreciated that it may be necessary to make routine variations to the dosage of either agent depending on the age and condition of the patient. The decision to begin the therapy will be based upon the appearance of the clinical manifestations of Thrombotic or Coagulopathic related disease, Inflammatory Disease or Repiratory Disease. Typical clinical manifestations are coughing, restricted breathing, obstructed airway, pain, fever, chills, tachycardia, tachypnea, altered mental state, hypothermia, hyperthermia, accelerated or repressed breathing or heart rates, increased or decreased white blood cell count, and hypotension. For Respiratory Disease diagnostic tests such as roetgenographic examination, bronchoscopy, lung biopsy, spirography (lung capacity, residual volume, flow rates, etc.) are used. These and other symptoms and diagnostic techniques are well known in the art as set out in standard references such as, Harrison's Principles of Internal medicine (ISBN 0-07-032370-4) 1994.

The decision to determine the length of therapy may be supported by standard clinical laboratory results from commercially available assays or instrumentation supporting the eradication of the symptoms defining Thrombotic or Coagulopathic related disease, Inflammatory or Respiratory Diseases. The method of the invention may be practiced by continuously or intermittently administering a therapeutically effective dose of the essential Protein C and TF antagonist ingredients for as long as deemed efficacious for the treatment of the episode. The administration can be conducted for up to a total of about 60 days with a preferred course of therapy lasting for up to 14 days. The therapy may be restarted upon the return of the Thrombotic or Coagulopathic related disease, Inflammatory or Respiratory disease.

Assays:

Factor VII biological activity

The biological activity of factor VIIa in blood clotting derives from its ability to (i) bind to tissue factor (TF) and (ii) catalyze the proteolytic cleavage of factor IX or factor X to produce activated factor IX or X (factor IXa or Xa, respectively).

Biological activity of factor VII polypeptides ("factor VII biological activity") may be quantified by measuring the ability of a preparation to promote blood clotting using factor VII-deficient plasma and thromboplastin, as described, e.g., in U.S. Patent No. 5,997,864. In this assay, biological activity is expressed as the reduction in clotting time relative to a control sample and is converted to "factor VII units" by comparison with a pooled human serum standard containing 1 unit/ml factor VII activity. Alternatively, factor VIIa biological activity may be quantified by

(i) Measuring the ability of factor VIIa or a factor VIIa -related polypeptide to produce activated factor X (factor Xa) in a system comprising TF embedded in a lipid membrane and factor X. (Persson et al., J. Biol. Chem. 272:19919-19924, 1997);

(ii) Measuring factor X hydrolysis in an aqueous system ("In Vitro Proteolysis Assay", see below);

(iii) Measuring the physical binding of factor VIIa or a factor VIIa -related polypeptide to TF using an instrument based on surface plasmon resonance (Persson, FEBS Letts. 413:359-363, 1997); and

(iv) Measuring hydrolysis of a synthetic substrate by factor VIIa and/or a factor VIIa -related polypeptide ("In Vitro Hydrolysis Assay", see below); and

(v) Measuring generation of thrombin in a TF-independent in vitro system.

In Vitro Hydrolysis Assay

Native (wild-type) factor VIIa and factor VIIa variant (both hereafter referred to as "factor VIIa") may be assayed for specific activities. They may also be assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). The chromogenic substrate D-Ile-Pro-Arg-*p*-nitroanilide (S-2288, Chromogenix, Sweden), final concentration 1 mM, is added to factor VIIa (final concentration 100 nM) in 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during a 20-minute incubation, after subtraction of the absorbance in a blank well containing no enzyme, is used to calculate the ratio between the activities of variant and wild-type factor VIIa:

$$\text{Ratio} = (A_{405\,nm} \text{ factor VIIa variant})/(A_{405\,nm} \text{ factor VIIa wild-type}).$$

Based thereon, factor VIIa variants with an activity comparable to or higher than native factor VIIa may be identified, such as, for example, variants where the ratio between the activity of the variant and the activity of native factor VII (wild-type FVII) is around, versus above 1.0.

The activity of factor VIIa or factor VIIa variants may also be measured using a physiological substrate such as factor X, suitably at a concentration of 100-1000 nM, where the factor

Xa generated is measured after the addition of a suitable chromogenic substrate (eg. S-2765). In addition, the activity assay may be run at physiological temperature.

In Vitro Proteolysis Assay

Native (wild-type) factor VIIa and factor VIIa variant (both hereafter referred to as "factor VIIa") are assayed in parallel to directly compare their specific activities. The assay is carried out in a microtiter plate (MaxiSorp, Nunc, Denmark). factor VIIa (10 nM) and factor X (0.8 microM) in 100 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl2 and 1 mg/ml bovine serum albumin, are incubated for 15 min. factor X cleavage is then stopped by the addition of 50 microL 50 mM Hepes, pH 7.4, containing 0.1 M NaCl, 20 mM EDTA and 1 mg/ml bovine serum albumin. The amount of factor Xa generated is measured by addition of the chromogenic substrate Z-D-Arg-Gly-Arg-p-nitroanilide (S-2765, Chromogenix, Sweden), final concentration 0.5 mM. The absorbance at 405 nm is measured continuously in a SpectraMax™ 340 plate reader (Molecular Devices, USA). The absorbance developed during 10 minutes, after subtraction of the absorbance in a blank well containing no FVIIa, is used to calculate the ratio between the proteolytic activities of variant and wild-type factor VIIa:

Ratio = (A405 nm factor VIIa variant)/(A405 nm factor VIIa wild-type).

Based thereon, factor VIIa variants with an activity comparable to or higher than native factor VIIa may be identified, such as, for example, variants where the ratio between the activity of the variant and the activity of native factor VII (wild-type FVII) is around, versus above 1.0.

Inhibition of FVIIa/phospholipids-embedded TF-catalyzed activation of FX by TF antagonists – FXa generation assay (assay 1):

In the following example all concentrations are final. Lipidated TF (10 pM), FVIIa (100 pM) and TF antagonist or FFR-rFVIIa (0 – 50 nM) in HBS/BSA (50 mM hepes, pH 7.4, 150 mM NaCl, 5 mM CaCl2,1 mg/ml BSA) are incubated 60 min at room temperature before FX (50 nM) is added. The reaction is stopped after another 10 min by addition of ½ volume stopping buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 20 mM EDTA). The amount of FXa generated is determined by adding substrate S2765 (0.6 mM, Chromogenix, and measuring absorbance at 405 nm continuously for 10 min. IC50 values for TF antagonist inhibition of FVIIa/lipidated TF-mediated activation of FX may be calculated. The IC50 value for FFR-rFVIIa is 51 +/- 26 pM in this assay.

Inhibition of FVIIa/cell surface TF-catalyzed activation of FX by TF antagonists – FXa generation assay (Assay 2):

In the following example all concentrations are final. Monolayers of human lung fibroblasts WI-38 (ATTC No. CCL-75) or human bladder carcinoma cell line J82 (ATTC No. HTB-1) or human keratinocyte cell line CCD 1102KerTr (ATCC no. CRL-2310) constitutively expressing TF

are employed as TF source in FVIIa/TF catalyzed activation of FX. Confluent cell monolayers in a 96-well plate are washed one time in buffer A (10 mM Hepes, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) and one time in buffer B (buffer A supplemented with with 1 mg/ml BSA and 5 mM Ca2+). FVIIa (1 nM), FX (135 nM) and varying concentrations of TF antagonist or FFR-rFVIIa in buffer B are simultaneously added to the cells. FXa formation is allowed for 15 min at 37°C. 50-µl aliquots are removed from each well and added to 50 µl stopping buffer (Buffer A supplemented with 10 mM EDTA and 1 mg/ml BSA). The amount of FXa generated is determined by transferring 50 µl of the above mixture to a microtiter plate well and adding 25 µl Chromozym X (final concentration 0.6 mM) to the wells. The absorbance at 405 nm is measured continuously and the initial rates of colour development are converted to FXa concentrations using a FXa standard curve. The IC50 value for FFR-rFVIIa is 1.5 nM in this assay.

Inhibition of 125I-FVIIa binding to cell surface TF by TF antagonists –
TF binding assay (Assay 3):

In the following example all concentrations are final. Binding studies are employed using the human bladder carcinoma cell line J82 (ATTC No. HTB-1) or the human keratinocyte cell line (CCD1102KerTr ATCC No CRL-2310) or NHEK P166 (Clonetics No. CC-2507) all constitutively expressing TF. Confluent monolayers in 24-well tissue culture plates are washed once with buffer A (10 mM Hepes, pH 7.45, 150 mM NaCl, 4 mM KCl, and 11 mM glucose) supplemented with 5 mM EDTA and then once with buffer A and once with buffer B (buffer A supplemented with with 1 mg/ml BSA and 5 mM Ca2+). The monolayers are preincubated 2 min with 100 µl cold buffer B. Varying concentrations of Mabs (or FFR-FVIIa) and radiolabelled FVIIa (0.5 nM 125I-FVIIa) are simultaneously added to the cells (final volume 200 µl). The plates are incubated for 2 hours at 4 °C. At the end of the incubation, the unbound material is removed, the cells are washed 4 times with ice-cold buffer B and lysed with 300 µl lysis buffer (200 mM NaOH, 1 % SDS and 10 mM EDTA). Radioactivity is measured in a gamma counter (Cobra, Packard Instruments). The binding data are analyzed and curve fitted using GraFit4 (Erithacus Software, Ltd., (U.K.). The IC50 value for FFR-rFVIIa is 4 nM in this assay.

Biosensor assay (Assay 4):

TF antagonists are tested on the Biacore instrument by passing a standard solution of the TF antagonist over a chip with immobilized TF. This is followed by different concentrations of sTF in 10 mM hepes pH 7.4 containing 150 mM NaCl, 10 mM CaCl2 and 0.0003 % polysorbate 20. Kd's are calculated from the sensorgrams using the integrated Biacore evaluation software.

Test for protein C activity:

A suitable assay for testing for Protein C anticoagulant and amidolytic activity and thereby selecting suitable protein C variants can be performed as described, for example, in Grinnell et al., 1987, Bio/Technology 5:1189-1192 (***"the Protein C assay"***).

The present invention is further illustrated by the following examples, which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realizing the invention in diverse forms thereof.

EXAMPLES

a. Preparation of Human Protein C

Recombinant human Protein C (r-hPC) was produced in Human Kidney 293 cells by techniques well known to the skilled artisan such as those set forth in Yan, U.S. Patent No. 4,981,952., the entire disclosure of which is herein incorporated by reference. The gene encoding human Protein C is disclosed and claimed in Bang et al., U.S. Patent No. 4,775,624, the entire disclosure of which is incorporated herein by reference. The plasmid used to express human Protein C in 293 cells was plasmid pLPC which is disclosed in Bang et al., U.S. Patent No. 4,992,373, the entire disclosure of which is incorporated herein by reference. The construction of plasmid pLPC is also described in European Patent Publication No. 0 445 939, the teachings of which are also incorporated herein by reference and in Grinnell et al., 1987, Bio/Technology 5:1189-1192. Briefly, the plasmid was transfected into 293 cells, then stable transformants were identified, subcultured and grown in serum-free media. After fermentation, cell-free medium was obtained by microfiltration.

The human Protein C was separated from the culture fluid by an adaptation of the techniques of Yan, U.S. Patent No. 4,981,952, the entire disclosure of which is herein incorporated by reference. The clarified medium was made 4 mM in EDTA before it was absorbed to an anion exchange resin (Fast-Flow Q, Pharmacia). After washing with 4 column volumes of 20 mm Tris, 200 mM NaCl, pH 7.4 and 2 column volumes of 20 mM Tris, 150 mM NaCl, pH 7.4, the bound recombinant human Protein C zymogen was eluted with 20 mM Tris, 150 mM NaCl, 10 mM CaC12, pH 7.4. The eluted protein was greater than 95% pure after elution as judged by SDS-polyacrylamide gel electrophoresis. Further purification of the protein was accomplished by making the protein 3 M in NaCl followed by adsorption to a hydrophobic interaction resin (Toyopearl Phenyl 650M, TosoHaas) equilibrated in 20 mM Tris, 3 M NaCl, 10 mM CaC12, pH 7.4. After washing with 2 column volumes of equilibration buffer without CaC12, the recombinant human Protein C was eluted with 20 mM Tris, pH 7.4. The eluted protein was prepared for activation by re-

moval of residual calcium. The recombinant human Protein C was passed over a metal affinity column (Chelex-100, Bio-Rad) to remove calcium and again bound to an anion exchanger (Fast Flow Q, Pharmacia). Both of these columns were arranged in series and equilibrated in 20 mM Tris, 1'50 mM NaCl, 5 mM EDTA, pH 7.4. Following loading of the protein, the Chelex-100 column was washed with one column volume of the same buffer before disconnecting it from the series. The anion exchange column was washed with 3 column volumes of equilibration buffer before eluting the protein with 0.4 M NaCl, 20 mM Tris-acetate, pH 6.5. Protein concentrations of recombinant human protein C and recombinant activated Protein C solutions were measured by UV 280 nm extinction EO.I%=1.85 or 1.95, respectively.

b. Activation of recombinant human Protein C

Bovine thrombin was coupled to Activated CH-Sepharose 4B (Pharmacia) in the presence of 50 mM HEPES, pH 7.5 at 4 OC. The coupling reaction was done on resin already packed into a column using approximately 5000 units thrombin/ml resin. The thrombin solution was circulated through the column for approximately 3 hours before adding MEA to a concentration of 0.6 ml/1 of circulating solution. The MEA-containing solution was circulated for an additional 10-12 hours to assure complete blockage of the unreacted amines on the resin. Following blocking, the thrombin-coupled resin was washed with 10 column volumes of 1 M NaCl, 20 mM Tris, pH 6.5 to remove all non-specifically bound protein, and was used in activation reactions after equilibrating in activation buffer. Purified rHPC was made 5 mM in EDTA (to chelate any residual calcium) and diluted to a concentration of 2 mg/ml with 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5. This material was passed through a thrombin column equilibrated at 37(C with 50 mM NaCl and either 20 irM Tris pH 7.4 or 20 mM Tris-acetate pH 6.5. The flow rate was adjusted to allow for approximately 20 min. of contact time between the rHPC and thrombin resin. The effluent was collected and immediately assayed for amidolytic activity. If the material did not have a specific activity (amidolytic) comparable to an established standard of aPC, it was recycled over the thrombin column to activate the rHPC to completion. This was followed by 1:1 dilution of the material with 20 mm buffer as above, with a pH of either 7.4 or 6.5 to keep the aPC at lower concentrations while it awaited the next processing step. Removal of leached thrombin from the aPC material was accomplished by binding the aPC to an anion exchange resin (Fast Flow Q, Pharmacia) equilibrated in activation buffer (either 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5) with 150 mM NaCl. Thrombin does not interact with the anion exchange resin under these conditions, but passes through the column into the sample application effluent. Once the aPC is loaded onto the column, a 2-6 column volume wash with 20 mM equilibration buffer is done before eluting the bound aPC with a step elution using 0.4 M NaCl in either 5 MM Tris-acetate, pH 6.5 or mM Tris, pH 7.4. Higher volume washes of the column facilitated more complete removal of the dodecapeptide. The material eluted from this column was stored either in a frozen solution (-20 OC) or as a lyophilised powder.

The anticoagulant activity of activated Protein C was determined by measuring the prolongation of the clotting time in the activated partial thromboplastin time (APTT) clotting assay. A standard curve was prepared in dilution buffer (1 mg/ml radioimmunoassay grade BSA, 20 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN3) ranging in Protein C concentration from 125 -1000 ng/ml. Samples were prepared at several dilutions in this concentration range. To each sample cuvette, 50 gl of cold horse plasma and 50 gl of reconstituted activated partial thromboplastin time reagent (APTT Reagent, Sigma) were added and incubated at 37 OC for 5 min. After incubation, 50 gl of the appropriate samples or standards were added to each cuvette. Dilution buffer was used in place of sample or standard to determine basal clotting time. The timer of the fibrometer (CoA Screener Hemostasis Analyzer, American Laboratory) was started immediately after the addition of 50 gl 37 (C 30 mM CaC12 to each sample or standard. Activated Protein C concentration in samples are calculated from the linear regression equation of the standard curve. Clotting times reported here are the average of a minimum of three replicates, including standard curve samples.

CLAIMS

1. A pharmaceutical composition comprising a tissue factor antagonist (TF antagonist) and protein C or a protein C-related polypeptide.

2. A composition according to claim 1, wherein the TF antagonist is a factor VII polypeptide which has a substantially reduced ability to catalyze factor X to factor Xa.

3. A composition according to claim 2, wherein the TF antagonist is a factor VII polypeptide catalytically inactivated in the active site.

4. A composition according to claim 3, wherein the TF antagonist is wild-type human factor VII catalytically inactivated in the active site.

5. A composition according to claim 3 or claim 4, wherein the factor VII polypeptide is catalytically inactivated in the active site with a chloromethyl ketone inhibitor independently selected from the group consisting of Phe-Phe-Arg chloromethyl ketone, Phe-Phe-Arg chloromethylketone, D-Phe-Phe-Arg chloromethyl ketone, D-Phe-Phe-Arg chloromethylketone Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, Phe-Pro-Arg chloromethylketone, D-Phe-Pro-Arg chloromethylketone, L-Glu-Gly-Arg chloromethylketone and D-Glu-Gly-Arg chloromethylketone, Dansyl-Phe-Phe-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethylketone, Dansyl-D-Phe-Phe-Arg chloromethyl ketone, Dansyl-D-Phe-Phe-Arg chloromethylketone, Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-D-Phe-Pro-Arg chloromethylketone, Dansyl-Phe-Pro-Arg chloromethylketone, Dansyl-D-Phe-Pro-Arg chloromethylketone, Dansyl-L-Glu-Gly-Arg chloromethylketone and Dansyl-D-Glu-Gly-Arg chloromethylketone.

6. A composition according to claim 1, wherein the TF antagonist is an antibody against TF.

7. A composition according to claim 6, wherein the TF antagonist is a monoclonal antibody.

8. A composition according to claim 7, wherein the TF antagonist is a fully human monoclonal antibody.

9. A composition according to claim 6, wherein the TF antagonist is selected from a list of: a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH I domains; a F(ab)2 or F(ab')2 fragment; a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the VH and CH1 domains; a Fv fragment

consisting of the VL and VH domains of a single arm of an antibody, a dAb fragment; an isolated complementarity determining region (CDR); and single chain Fv (scFv).

10. A composition according to any one of claims 1 to 9, wherein the protein C or protein C-related polypeptide is human protein C.

11. A composition according to claim 10, wherein said protein C is activated human protein C.

12. A composition according to claim 10 or claim 11, wherein said protein C is recombinantly made.

13. A composition according to any one of claims 1 to 9, wherein the protein C or protein C-related polypeptide is a protein C-related polypeptide.

14. A composition according to claim 13, wherein said protein C-related polypeptide is a protein C amino acid sequence variant.

15. A composition according to claim 13 or claim 14, wherein the ratio between the activity of said protein C-related polypeptide and the activity of native human plasma protein C (wild-type PC) is at least about 1.25 when tested in the "protein C activity assay" as described in the present description.

16. A composition according to any one of claims 1 to 15, wherein the TF antagonist and the protein C or protein C-related polypeptide are present in a ratio by mass of between about 100:1 and about 1.100 (w/w factor VII:protein C)

17. A composition according to any one of claims 1 to 16, wherein the composition further comprises pharmaceutically acceptable excipients suitable for injection or infusion, in particular injection.

18. Use of a TF antagonist in combination with protein C or a protein C-related polypeptide for the manufacture of a medicament for treating Thrombotic and Coagulopathic related diseases, Respiratory diseases and Inflammatory diseases.

19. Use according to claim 18, wherein the medicament is for treatment of a disease selected from the list of: deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke,

tumour metastasis, inflammation, septic chock, hypotension, acute lung injury (ALI), Acute Respiratory Distress Syndromr (ARDS), pulmonary embolism, disseminated intravascular coagulation (DIC), sepsis, systemic inflammatory response syndrome (SIRS), vascular restenosis, platelet deposition, myocardial infarction, angiogenesis, or the prophylactic treatment of mammals with atherosclerotic vessels at risk for thrombosis; asthma, bronchitis, idiopathic pulmonary fibrosis, pneumonia, pulmonary edema, pulmonary obstructive disease, endotoxin induced lung damage, non cell lung cancer; inflammatory bowel disease, pancreatitis, trauma-induced shock, bronchial asthma, allergic rhinitis, rheumatoid arthritis, cystic fibrosis, stroke, acute bronchitis, chronic bronchitis, acute bronchiolitis, chronic bronchiolitis, osteoarthritis, gout, spondylarthropathris, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, enterapathric spondylitis, juvenile arthropathy or juvenile ankylosing spondylitis, reactive arthropathy, infectious or post-infectious arthritis, gonoccocal arthritis, tuberculous arthritis, viral arthritis, fungal arthritis, syphilitic arthritis, Lyme disease, arthritis associated with "vasculitic syndromes," polyarteritis nodosa, hypersensitivity vasculitis, Luegenec's granulomatosis, polymyalgin rheumatica, joint cell arteritis, calcium crystal deposition arthropathris, pseudo gout, non-articular rheumatism, bursitis, tenosynomitis, epicondylitis (tennis elbow), carpal tunnel syndrome, repetitive use injury (typing), miscellaneous forms of arthritis, neuropathic joint disease (charco and joint), hemarthrosis (hemarthrosic), Henoch-Schonlein Purpura, hypertrophic osteoarthropathy, multicentric reticulohistiocytosis, arthritis associated with certain diseases, surcoilosis, hemochromatosis, sickle cell disease and other hemoglobinopathries, hyperlipoproteineimia, hypogammaglobulinemia, hyperparathyroidism, acromegaly, familial Mediterranean fever, Behat's Disease, systemic lupus erythrematosis, relapsing, and multiple organ failure resulting from any of the preceding pathologic processes.

20. Use according to claim 19, wherein the disease is selected from the list of: systemic inflammatory response syndrome, asthma, bronchitis, acute lung injury, acute resporatory distress syndrome, idiopathic pulmonary fibrosis, pneumonia, pulmonary edema, pulmonary obstructive disease, endotoxin induced lung damage, non cell lung cancer; inflammatory bowel disease, sepsis, septic shock, acute respiratory distress syndrome, pancreatitis, trauma-induced shock, bronchial asthma, allergic rhinitis, rheumatoid arthritis, cystic fibrosis, stroke, acute bronchitis, chronic bronchitis, acute bronchiolitis, chronic bronchiolitis, osteoarthritis, gout, spondylarthropathris, ankylosing spondylitis, Reiter's syndrome, psoriatic arthropathy, enterapathric spondylitis, juvenile arthropathy or juvenile ankylosing spondylitis, reactive arthropathy, infectious or post-infectious arthritis, gonoccocal arthritis, tuberculous arthritis, viral arthritis, fungal arthritis, syphilitic arthritis, Lyme disease, arthritis associated with "vasculitic syndromes," polyarteritis nodosa, hypersensitivity vasculitis, Luegenec's granulomatosis, polymyalgin rheumatica, joint cell arteritis, calcium crystal deposition arthropathris, pseudo gout, non-articular rheumatism, bursi-

tis, tenosynomitis, epicondylitis (tennis elbow), carpal tunnel syndrome, repetitive use injury (typing), miscellaneous forms of arthritis, neuropathic joint disease (charco and joint), hemarthrosis (hemarthrosic), Henoch-Schonlein Purpura, hypertrophic osteoarthropathy, multicentric reticulohistiocytosis, arthritis associated with certain diseases, surcoilosis, hemochromatosis, sickle cell disease and other hemoglobinopathries, hyperlipoproteineimia, hypogammaglobulinemia, hyperparathyroidism, acromegaly, familial Mediterranean fever, Behat's Disease, systemic lupus erythrematosis, relapsing, and multiple organ failure resulting from any of the preceding pathologic processes.

21. Use according to claim 19, wherein the disease is selected from the list of: deep venous thrombosis, arterial thrombosis, post surgical thrombosis, coronary artery bypass graft (CABG), percutaneous transdermal coronary angioplastry (PTCA), stroke, tumour metastasis, inflammation, septic chock, hypotension, acute lung injury (ALI), Acute Respiratory Distress Syndromr (ARDS), pulmonary embolism, disseminated intravascular coagulation (DIC), sepsis, systemic inflammatory response syndrome (SIRS), vascular restenosis, platelet deposition, myocardial infarction, angiogenesis, or the treatment of mammals with atherosclerotic vessels at risk for thrombosis, and multiple organ failure resulting from any of the preceding pathologic processes.

22. Use according to any one of claims 19 to 21, wherein the disease is one or more of systemic inflammatory response syndrome, acute lung injury, acute respiratory distress syndrome, disseminated intravascular coagulation, sepsis, or multiple organ failure in association with any of the preceeding syndromes.

23. Use according to any one of claims 18 to 22, wherein the medicament is formulated for injection or infusion, in particular injection.

24. Use according to any one of claims 18 to 23, wherein the medicament is in single-dosage form.

25. Use according to any one of claims 18 to 23, wherein the medicament is prepared in the form of a first unit dosage form comprising a preparation of a TF antagonist and a second unit dosage form comprising a preparation of protein C or a protein C-related polypeptide.

26. A method for treating Thrombotic or Coagulapatic diseases, Respiratory diseases and Inflammatory diseases in a subject, the method comprising administering to a subject in need thereof a first amount of a preparation of a TF antagonist and a second amount of a preparation of protein C or a protein C-related polypeptide, wherein the first and second amount together

are effective to treat Thrombotic or Coagulapatic diseases, Respiratory diseases and Inflammatory diseases.

27. Method according to claim 26, wherein the TF antagonist and the protein C or protein C-related polypeptide are administered in single-dosage form.

28. Method according to claim 26, wherein the TF antagonist and the protein C or protein C-related polypeptide are administered in the form of a first dosage form comprising a preparation of a TF antagonist and a second dosage form comprising a preparation of protein C or a protein C-related polypeptide.

Novo Nordisk A/S

ABSTRACT

The present invention relates to a composition comprising a TF antagonist and protein C or a protein C-related polypeptide, and the use thereof for treating Thrombotic or Coagulopathic relatedl diseases, Respiratory diseases and Inflammatory diseases.

SEQUENCE LISTING

SEQ ID NO:1 (The amino acid sequence of native human coagulation Factor VII):

```
Ala-Asn-Ala-Phe-Leu-GLA-GLA-Leu-Arg-Pro-Gly-Ser-Leu-GLA-Arg-GLA-Cys-Lys-
                  5                  10                  15

GLA-GLA-Gln-Cys-Ser-Phe-GLA-GLA-Ala-Arg-GLA-Ile-Phe-Lys-Asp-Ala-GLA-Arg-
     20                  25                  30                  35

Thr-Lys-Leu-Phe-Trp-Ile-Ser-Tyr-Ser-Asp-Gly-Asp-Gln-Cys-Ala-Ser-Ser-Pro-
             40                  45                  50

Cys-Gln-Asn-Gly-Gly-Ser-Cys-Lys-Asp-Gln-Leu-Gln-Ser-Tyr-Ile-Cys-Phe-Cys-
 55                  60                  65                  70

Leu-Pro-Ala-Phe-Glu-Gly-Arg-Asn-Cys-Glu-Thr-His-Lys-Asp-Asp-Gln-Leu-Ile-
         75                  80                  85                  90

Cys-Val-Asn-Glu-Asn-Gly-Gly-Cys-Glu-Gln-Tyr-Cys-Ser-Asp-His-Thr-Gly-Thr-
                 95                 100                 105

Lys-Arg-Ser-Cys-Arg-Cys-His-Glu-Gly-Tyr-Ser-Leu-Leu-Ala-Asp-Gly-Val-Ser-
    110                 115                 120                 125

Cys-Thr-Pro-Thr-Val-Glu-Tyr-Pro-Cys-Gly-Lys-Ile-Pro-Ile-Leu-Glu-Lys-Arg-
            130                 135                 140

Asn-Ala-Ser-Lys-Pro-Gln-Gly-Arg-Ile-Val-Gly-Gly-Lys-Val-Cys-Pro-Lys-Gly-
145                 150                 155                 160

Glu-Cys-Pro-Trp-Gln-Val-Leu-Leu-Leu-Val-Asn-Gly-Ala-Gln-Leu-Cys-Gly-Gly-
        165                 170                 175                 180

Thr-Leu-Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala-His-Cys-Phe-Asp-Lys-Ile-
                185                 190                 195

Lys-Asn-Trp-Arg-Asn-Leu-Ile-Ala-Val-Leu-Gly-Glu-His-Asp-Leu-Ser-Glu-His-
    200                 205                 210                 215

Asp-Gly-Asp-Glu-Gln-Ser-Arg-Arg-Val-Ala-Gln-Val-Ile-Ile-Pro-Ser-Thr-Tyr-
            220                 225                 230

Val-Pro-Gly-Thr-Thr-Asn-His-Asp-Ile-Ala-Leu-Leu-Arg-Leu-His-Gln-Pro-Val-
235                 240                 245                 250

Val-Leu-Thr-Asp-His-Val-Val-Pro-Leu-Cys-Leu-Pro-Glu-Arg-Thr-Phe-Ser-Glu-
        255                 260                 265                 270

Arg-Thr-Leu-Ala-Phe-Val-Arg-Phe-Ser-Leu-Val-Ser-Gly-Trp-Gly-Gln-Leu-Leu-
                275                 280                 285

Asp-Arg-Gly-Ala-Thr-Ala-Leu-Glu-Leu-Met-Val-Leu-Asn-Val-Pro-Arg-Leu-Met-
    290                 295                 300                 305 306
```

```
Thr-Gln-Asp-Cys-Leu-Gln-Gln-Ser-Arg-Lys-Val-Gly-Asp-Ser-Pro-Asn-Ile-Thr-
            310                 315                 320

Glu-Tyr-Met-Phe-Cys-Ala-Gly-Tyr-Ser-Asp-Gly-Ser-Lys-Asp-Ser-Cys-Lys-Gly-
325                 330                 335                 340

Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr-Arg-Gly-Thr-Trp-Tyr-Leu-Thr-Gly-
        345                 350                 355                 360

Ile-Val-Ser-Trp-Gly-Gln-Gly-Cys-Ala-Thr-Val-Gly-His-Phe-Gly-Val-Tyr-Thr-
                365                 370                 375

Arg-Val-Ser-Gln-Tyr-Ile-Glu-Trp-Leu-Gln-Lys-Leu-Met-Arg-Ser-Glu-Pro-Arg-
    380                 385                 390                 395

Pro-Gly-Val-Leu-Leu-Arg-Ala-Pro-Phe-Pro
            400                 405 406
```